PCT/AU2004/000866

Patent Office
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I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2003903251 for a patent by BIOTRON LIMITED as filed on 26 June 2003.

WITNESS my hand this
Seventh day of July 2004

J Billingsley

JULIE BILLINGSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

ANTIVIRAL COMPOUNDS AND METHODS

FIELD OF INVENTION

The present invention relates to methods for retarding, reducing or otherwise inhibiting viral growth and/or functional activity. The invention also relates to compounds and compositions suitable for use in the methods.

BACKGROUND OF THE INVENTION

Currently, there is a great need for the development of new treatments that are effective against viral infections, particularly against viral infections which are associated with high morbidity and mortality, and which impact on sizable populations. Treatments currently available are inadequate or ineffective in large proportions of infected patients.

For example, in ameliorating AIDS symptoms and prolonging life expectancy, a measure of success has been achieved with drugs targeting the viral reverse transcriptase and protease enzymes (Miller and Sarver, 1997; Mitsuya, 1992; Moore, 1997; and Thomas and Brady, 1997). However, no single treatment method is completely effective against HIV infection. (Barry et al, 1998; Deeks, 1998; Miles, 1997; Miles, 1998; Moyle et al, 1998; Rachlis and Zarowny, 1998; Veil et al, 1997; Volberding and Deeks, 1998; and Volberdin, 1998).

PCT application PCT/AU99/00872 describes the use of compounds 5-(N,N-hexamethylene)-amiloride and 5-(N,N-dimethyl)-amiloride in the treatment of HIV infection.

Another example is the Hepatitis C virus (HCV). This is a significant human pathogen in terms of both cost to human health and associated economic costs. HCV causes chronic hepatitis and cirrhosis and is the leading indicator for liver replacement surgery. In 2002 the Centre for Disease Control and Prevention estimated that more than 4 million people were infected in the USA alone and that approximately 8,000 to 10, 000 die as a result of chronic HCV infection yearly. There is no known cure or vaccine. More effective pharmacological agents are urgently required.

To improve the prospect of treating and preventing viral infections, there is an on-going need to identify molecules capable of inhibiting various aspects of the viral life cycle.

It is an object of the present invention to overcome or ameliorate at least one of the disadvantages of the prior art, or to provide a useful alternative.

SUMMARY OF THE INVENTION

The inventors have surprisingly found that although the drug amiloride has no detrimental effect on viral replication, amiloride analogues, in which the amino group located at the 5 position and/or the chloride located at the 6-position of the pyrazine has been substituted, and/or the amino group located at the 3-position of the pyrazine has been substituted, or amiloride analogues in which one or both of the guanidyl amino groups has been substituted, have anti-viral activity against different viruses.

Further, and without wishing to be bound by any particular theory or mechanisms of action, despite current dogma it appears possible that viral replication can be retarded by inhibiting or otherwise down-regulating ion channel functioning. Thus, the negative impact of the compounds of the present invention on viral replication may be mediated by the inhibition or otherwise down-regulation of a membrane ion channel relied upon by the virus for replication. This membrane ion channel may be a viral membrane ion channel (exogenous to the host cell) or a host cell ion channel induced as a result of viral infection (endogenous to the host cell). As an example, the compounds of the present invention may inhibit Vpu or p7 function and thereby inhibit the continuation of the HIV and HCV life cycle. These concepts are covered in certain preferred embodiments of the present invention, described herein.

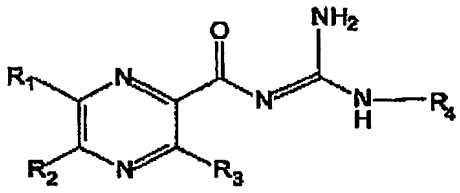
The present invention is concerned with novel antiviral compounds comprising a guanidyl moiety and in particular to novel antiviral amiloride analogues and/or derivatives. However, it does not include in its scope the use of compounds 5-(N,N-hexamethylene)amiloride and 5-(N,N-dimethyl)-amiloride for retarding, reducing or otherwise inhibiting viral growth and/or functional activity of HIV.

Accordingly, a first aspect of the present invention provides an antiviral compound comprising a guanidyl moiety.

According to a second aspect, the invention provides an antiviral amiloride analogue and or/derivative.

Preferably, the compounds of the invention are capable of reducing, retarding or otherwise inhibiting viral growth and/or replication.

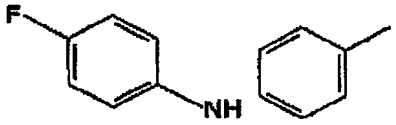
Preferably, the amiloride analogues and or derivatives, and other compounds containing a guanidyl moiety comprise the structure

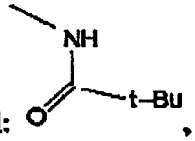


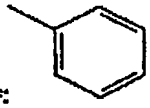
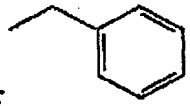
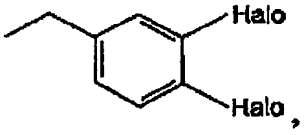
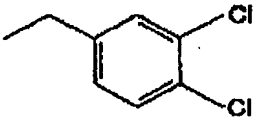
wherein the substituents at the R positions may or maynot be the same, and

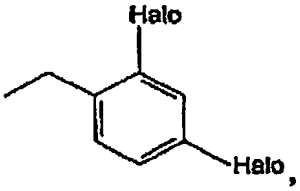
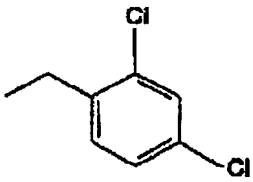
R_1 = halo, aryl, substituted aryl, phenyl, or substituted phenyl;

R_2 = amine, aryl, substituted aryl, phenyl, substituted phenyl, hexamethylene, PrS, N-methyl-N-isobutyl, N-ethyl –N-isopropyl, benzyl ; N-methyl-N-guanidinocarbonyl-methyl, N,N-dimethyl, N,N-diethyl, tert-butylamino, halo-aniline, fluoro-aniline



R_3 = hydroxy, alkyloxy, methoxy, N,3-dimethylbutanamyl:  ,

R_4 =:  or  or,  , 

 ,  ,

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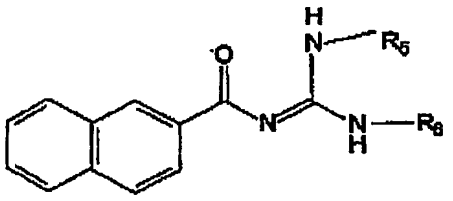
The following antiviral compounds comprising a guanidyl moiety are also encompassed within the scope of the present invention:



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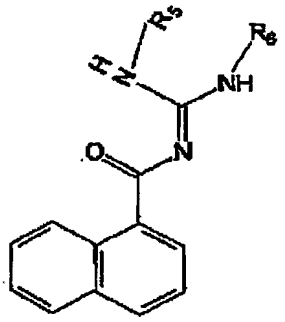

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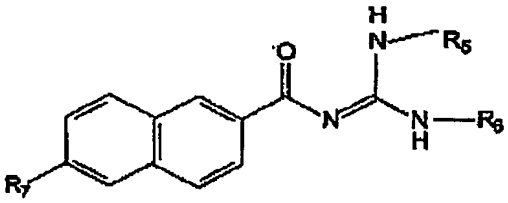
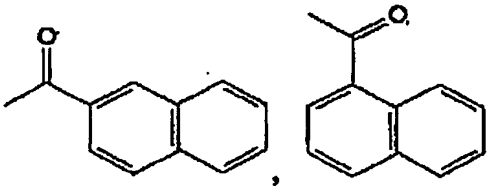


wherein the substitutents at the R positions may or may not be the same, and

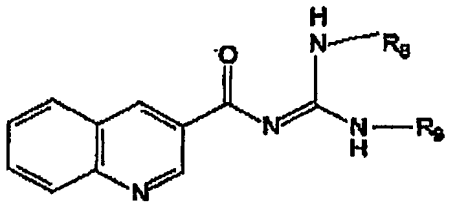
R_5= H, aryl, substituted aryl, phenyl, or substituted phenyl;

R_6 = H, aryl, substituted aryl, phenyl, substituted phenyl, napthoyl,



R_7 = alkyloxy, methoxy;

or, the structure

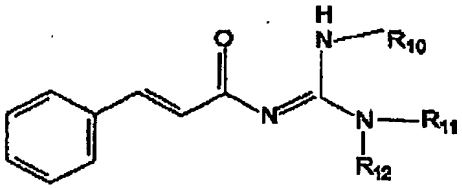


wherein the substitutents at the R positions may or may not be the same, and

R_8 = aliphatic or aromatic substituents;

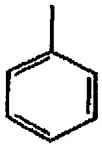
R_9 = aliphatic or aromatic substituents;

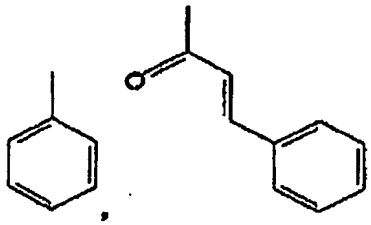
or, the structure



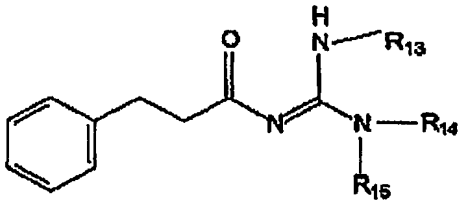
wherein the substitutents at the R positions may or may not be the same, and

R_{10} = H, aryl, phenyl, cinnamoyl;

R_{11} = H, alkyl, aryl, phenyl, cinnamoyl; 

R_{12} = H, alky, aryl, phenyl, cinnamoyl,  ,

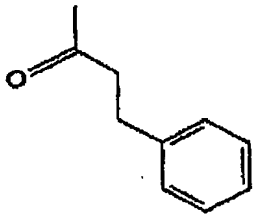
or, the structure



wherein the substitutents at the R positions may or may not be the same, and

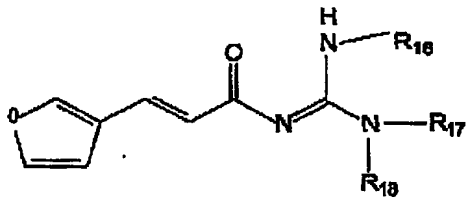
R_{13} = H; alkyl, or phenyl

R_{14} = H, alkyl, phenyl, or substituted phenyl, -



R_{15} = H, or

or, the structure



wherein the substitutents at the R positions may or may not be the same, and

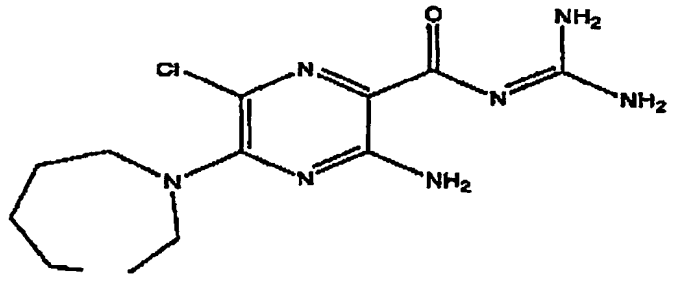
R_{16} = H, alkyl, substituted alkyl, phenyl, or substituted phenyl;

R_{17} = H, alkyl, substituted alkyl, phenyl, or substituted phenyl;

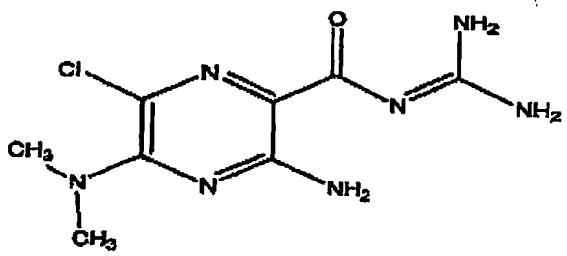
R_{18} = H, alkyl, substituted alkyl, phenyl, or substituted phenyl;

The compounds of the invention include the following:

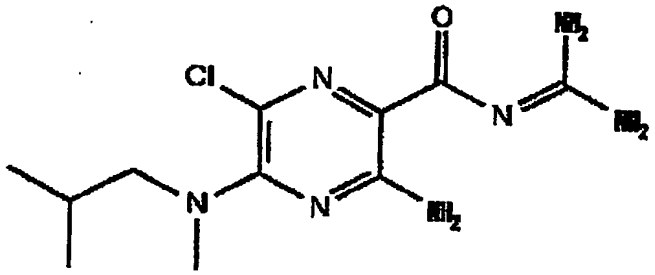
5-(N,N-hexamethylene)amiloride



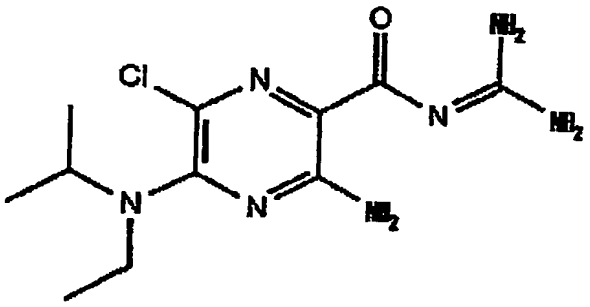
5-(N,N-Dimethyl)amiloride hydrochloride



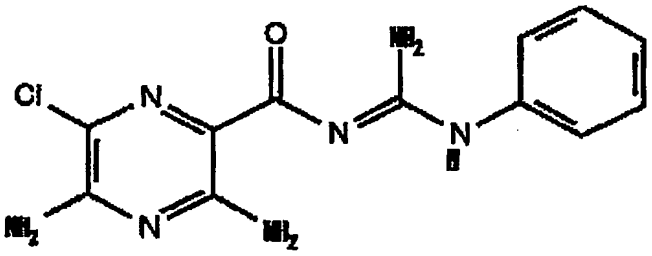
5-(N-methyl-N-isobutyl)amiloride comprising the structure



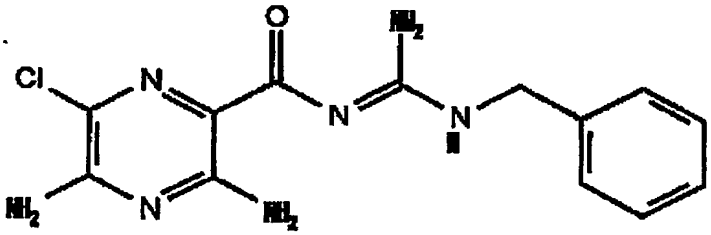
5-(N-ethyl-N-isopropyl)amiloride (herein referred to as EIPA), comprising the structure



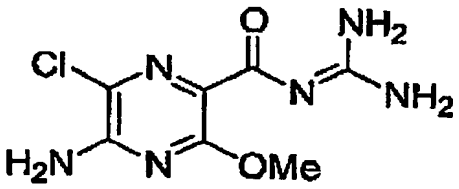
N-(3,5-Diamino-6-chloro-pyrazine-2-carbonyl)-N'-phenyl-guanidine, comprising the structure



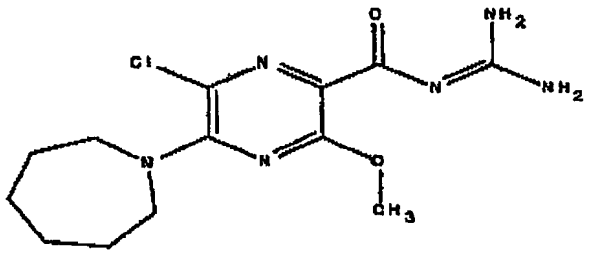
N-Benzyl –N'-(3,5-diamino-6-chloro-pyrzine-2-carbonyl)-guanidine, comprising the structure



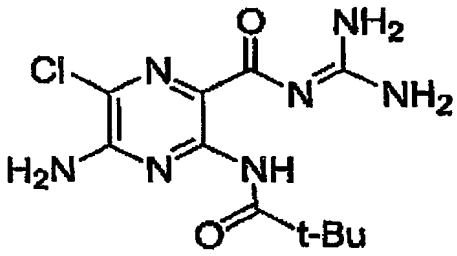
3-methoxy amiloride comprising the structure



3-methoxy-5-(N,N-Hexamethylene)-amiloride comprising the structure



3-(N-2,2 -dimethyl propanal)amiloride comprising the structure



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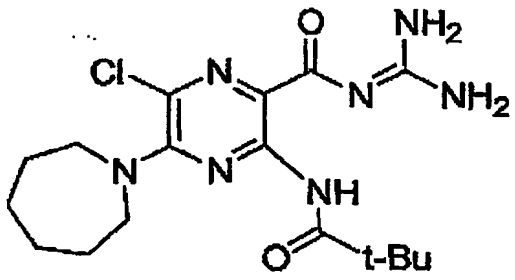
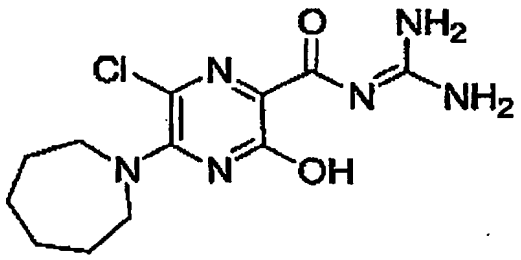
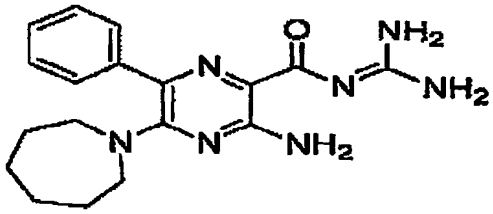
3-(N-2,2 -dimethyl propanal)-5-N-hexamethylene amiloride comprising the structure



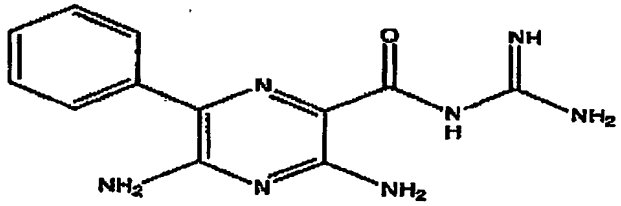
3-hydroxy-5-hexamethyleneimino-amiloride comprising the structure



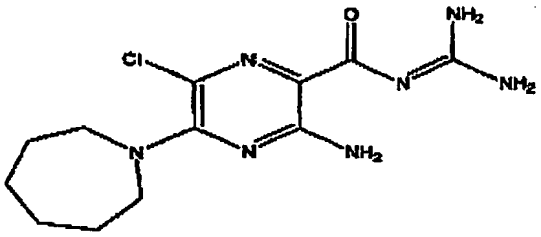
Hexamethyleneimino-6-phenyl-2-pyraxinecarboxamide comprising the structure



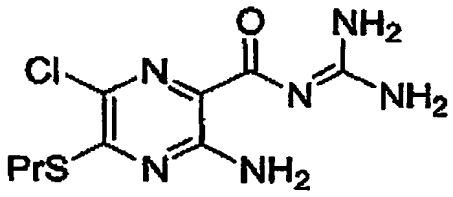
N-amidino-3,5-diamino-6-phenyl-2-pyrazinecarboxamide comprising the structure



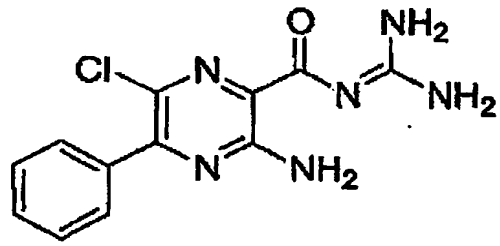
5-(N,N-hexamethylene)amiloride comprising the structure



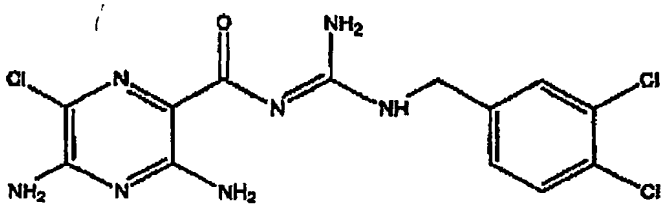
5-propyl-sulfide amiloride comprising the structure



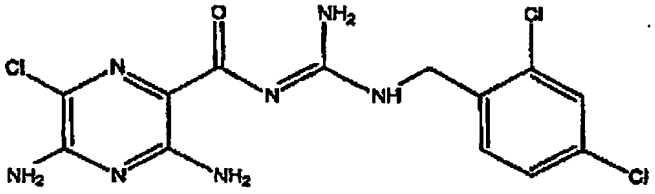
N-amidino-3-amino-5-phenyl-6-chloro-2-pyrazinecarboxamide comprising the structure



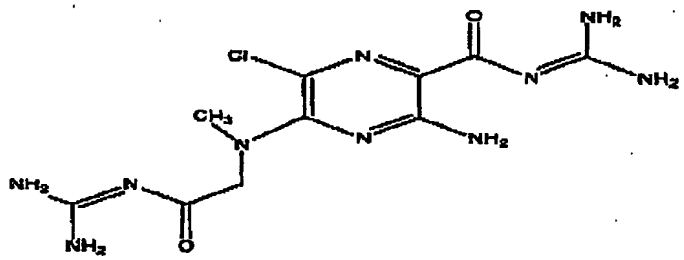
3'4 DichloroBenzamil comprising the structure



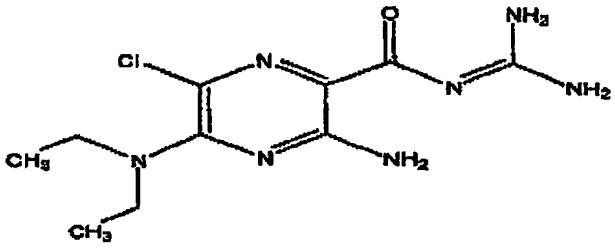
2'4 DichloroBenzamil HCl comprising the structure



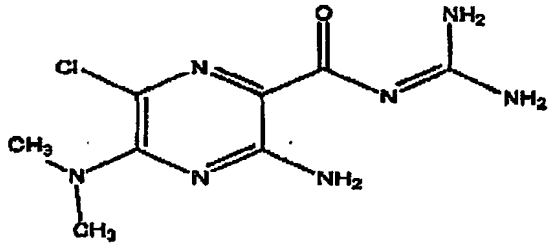
5 5-(N-methyl-N-guanidinocarbonyl-methyl)amiloride comprising the structure



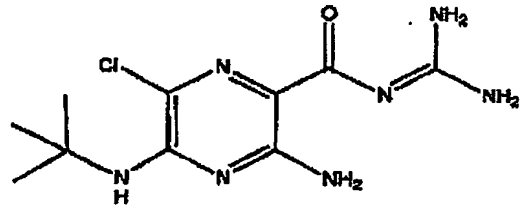
5-(N,N-Diethyl)amiloride hydrochloride comprising the structure



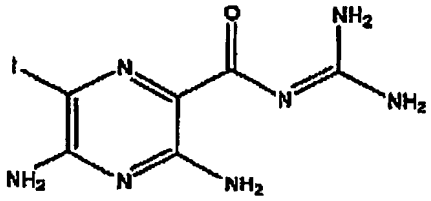
5-(N,N-Dimethyl)amiloride hydrochloride comprising the structure



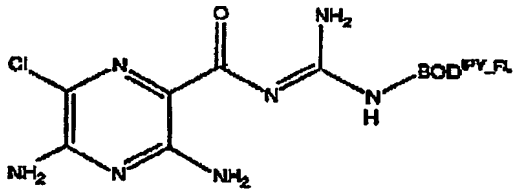
5-tert-butylamino-amiloride comprising the structure



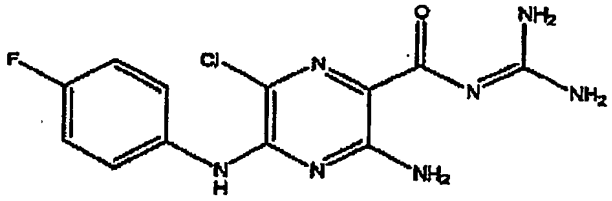
6-Iodoamiloride comprising the structure



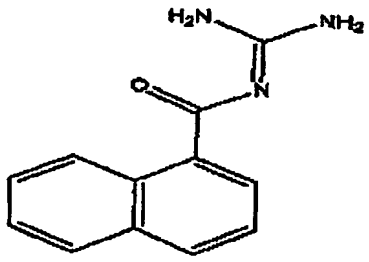
Bodipy-FL Amiloride comprising the structure



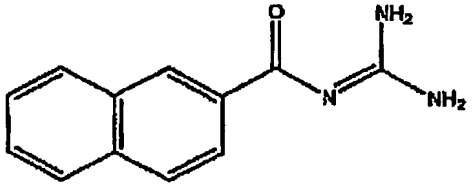
5-(4-fluorophenyl)amiloride comprising the structure



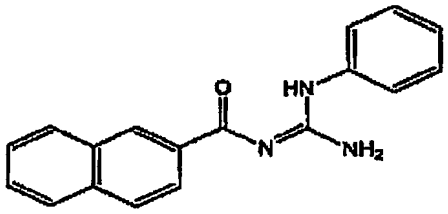
1-napthoylguanidine comprising the structure



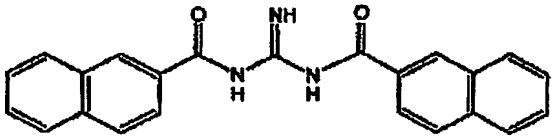
2-napthoylguanidine comprising the structure



N-(2-napthoyl)-N'-phenylguanidine comprising the structure



N,N'-bis(2-napthoyl)guanidine comprising the structure



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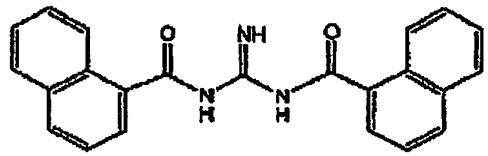
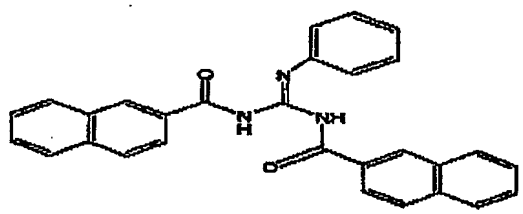
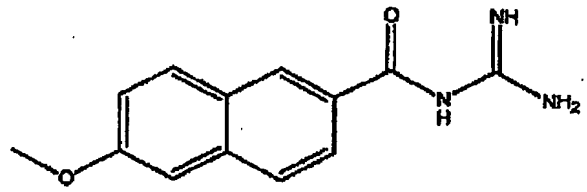


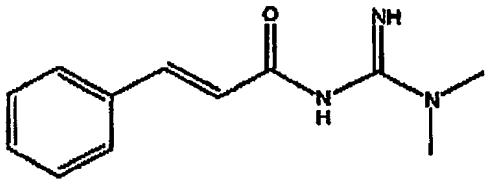
N,N'-bis(2-napthoyl)-N"-phenylguanidine comprising the structure



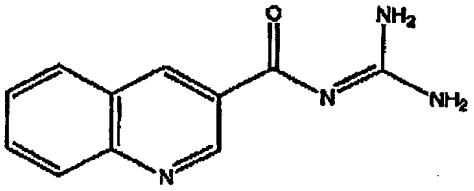
6-methoxy-2-naphthoylguanidine comprising the structure



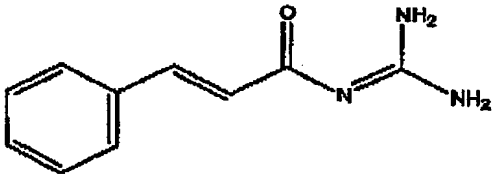
N-Cinnamoyl-N',N'-dimethylguanidine comprising the structure



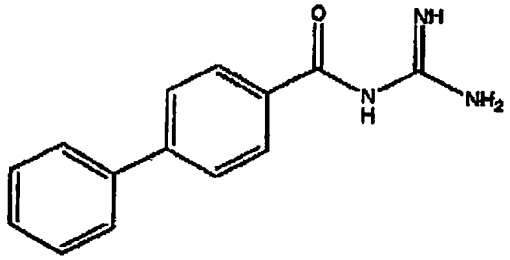
3-quinolinoylguanidine comprising the structure



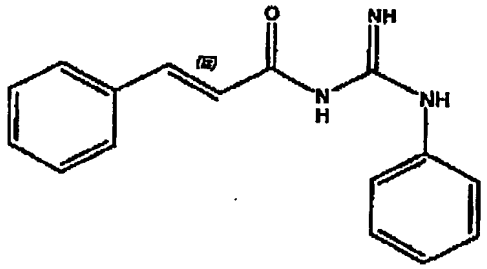
cinnamoylguanidine comprising the structure



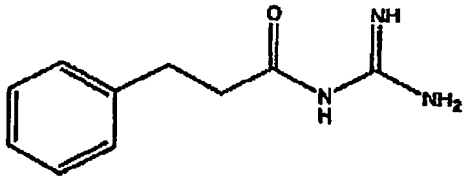
4-phenylbenzoylguanidine comprising the structure



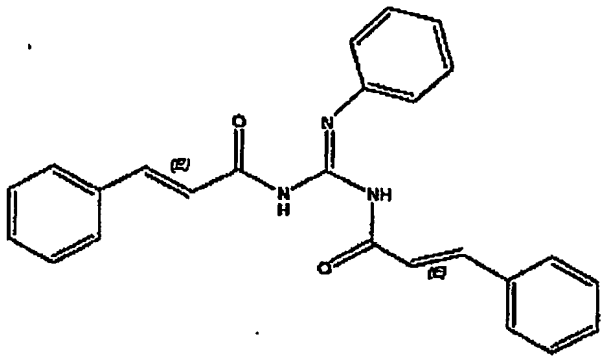
N-(cinnamoyl)-N'phenylguanidine comprising the structure



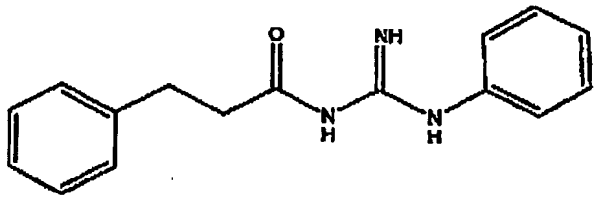
(3-phenylpropanoyl)guanidine comprising the structure



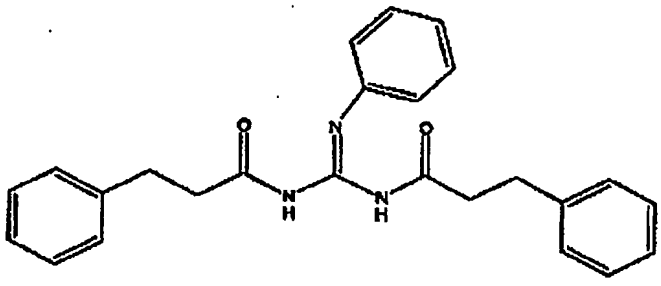
N,N'-bis-(cinnamoyl)-N"-phenylguanidine comprising the structure



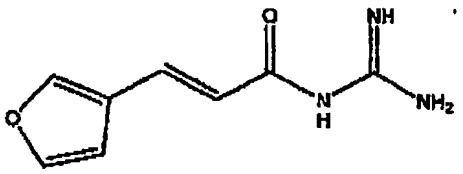
N-(3-phenylpropanoyl)-N'-phenylguanidine comprising the structure



N,N'-bis(3phenylpropanoyl)-N"-phenylguanidine comprising the structure



trans-3-furanacryoylguanidine comprising the structure



Preferably, the antiviral activity of the compounds of the invention is towards viruses such as, for example, HIV and HCV.

According to a third aspect of the present invention, there is provided a pharmaceutical composition comprising a compound according to any one of the previous aspects, and optionally one or more pharmaceutical acceptable carriers or derivatives.

According to a fourth aspect, there is provided a method for reducing, retarding or otherwise inhibiting growth and/or replication of a virus comprising contacting a cell infected with said virus or exposed to said virus with a compound according the first or second aspect or a composition according to the third aspect.

According to a fifth aspect, there is provided a method for preventing the infection of a cell exposed to a virus comprising contacting said cell with a compound according to the first or second aspect or a compositions according to the third aspect.

According to a sixth aspect, there is provided a method of down regulating a membrane ion channel functional activity in a cell infected with a virus, comprising contacting said cell with a compound according to the first or second aspect or a composition according to the third aspect, wherein said membrane ion channel is endogenous to the cell or exogenous to the cell.

Preferably, the membrane ion channel of which functional activity is compromised is that which the Human Immunodeficiency Virus (HIV) or the Hepatitis C virus (HCV) utilises for replication and includes the HIV membrane ion channel Vpu and the HCV membrane ion channel P7, respectively.

According to a seventh aspect of the invention, there is provided a method for the therapeutic or prophylactic treatment of a subject infected with or exposed to a virus comprising the administration of a pharmaceutical composition comprising a compound according to the first or second aspect or a composition according to the third aspect, to a subject in need of said treatment.

According to an eighth aspect, the invention provides the use of a compound according to the first or second aspect, in the manufacture of a medicament for the therapeutic or prophylactic treatment of a subject infected with or exposed to a virus.

Preferably, and unless otherwise stated, viral infection is that of, for example, infection with HIV or HCV.

Unless the context clearly requires otherwise, throughout the description and the claims, the words 'comprise', 'comprising', and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of plasmids used for expression of Vpu in *E. coli*. **A.** The amino acid sequence (< 400 > 1) encoded by the *vpu* open reading frame (ORF) generated by PCR from an HIV- 1 strain HXB2 cDNA clone. The *vpu* ORF was cloned in-frame at the 3' end of the GST gene in p2GEX to generate p2GEXVpu **(B)**. It was subsequently cloned into pPL451 to produce the plasmid pPL + Vpu (C).

Figure 2 is a photographic representation of the expression and purification of Vpu in *E. coli*. **A.** Western blotting after SDS-PAGE was used to detect expressed Vpu in *E. coli* extracts. **Lanes 1-4** contain samples, at various stages of purity, of Vpu expressed from p2GEXVpu: **lane 1,** GST-Vpu fusion protein isolated by glutathione-agarose affinity chromatography; **lane 2,** Vpu liberated from the fusion protein by treatment with thrombin; **lane 3,** Vpu purified by HPLC anion exchange chromatography; **lane 4,** Vpu after passage through the immunoaffinity column. **Lanes 5 and 6,** membrane vesicles prepared from 42'C induced cells containing pPL+Vpu or pPL451, respectively.

B. Silver stained SDS-PAGE gel: lane 1, Vpu purified by HPLC anion exchange chromatography; lane 2, Vpu after passage through the immunoaffinity column.

Figure 3 is a graphical representation of ion channel activity observed after exposure of lipid bilayers to aliquots containing purified Vpu. In A and B, the CIS chamber contained 500mM NaCl and the TRANS chamber contained 50mM NaCl; both solutions were buffered at pH 6.0 with 10 mM MES. B shows a current versus voltage curve generated from data similar to that shown in A.

Figure 4 is a photographic representation of bacterial cross-feeding assays. For all plates, the Met^-, Pro^- auxotrophic strain was used to seed a soft agar overlay. Plates A and B contain minimal drop-out medium minus proline; in plate C the medium was minus methionine. To control for viability of the cells in the background lawn, the discs labelled P and M contained added proline or methionine, respectively. The discs labelled C and V were inoculated with Met^+, Pro^+ *E. coli* cells containing the plasmids pPL451 or pPL+Vpu, respectively. Plates were incubated at 37°C (A and C) or 30°C (B) for two days and photographed above a black background with peripheral illumination from a fluorescent light located below the plate. The images were recorded on a Novaline video gel documentation system. Light halos around the discs labelled P or M on all plates and around the disc labelled V on plate A indicate growth of the background lawn strain.

Figure 5 is a graphical representation of the screening of drugs for potential Vpu channel blockers. The photograph shows a section of a minimal medium-lacking adenine - agarose plate onto which a lawn of XL-1-blue *E. coli* cells containing the Vpu expression plasmid pPLVpu has been seeded. Numbers 6-11 are located at the sites of application of various drugs being tested, which were applied in 3µl drops and allowed to soak into the agarose. The plate was then incubated at 37°C for 48hr prior to being photographed. The background grey shade corresponds to areas of no bacterial growth. The bright circular area around " 10 " represents bacterial cell growth as a result of application of adenine at that location (positive control). The smaller halo of bacterial growth around "9" is due to the application of 5-(N,N-hexamethylene)-amiloride at that location.

Figure 6 is a graphical representation of the inhibition of Vpu ion channel activity by 5(N,N-hexamethylene)-amiloride (HMA) in planar lipid bilayers. The three traces

represent typical Vpu channel activity observed in the presence of the indicated concentrations of HMA. The solid line indicates the zero current level. Mean currents (+/- variance), calculated for continuous channel recordings of at least 30 seconds duration, are plotted in the graph for each of the three drug concentrations.

Figure 7 is a graphical representation of the effect of HMA on HIV virion production in monocytes and monocyte-derived macrophages. HIV in culture supernatants was assayed at various days post-infection by detection of p24 antigen using a quantitative ELISA method. Solid black bars represent HIV-infected cells exposed to 10μM HMA. Hatched bars are control cells not exposed to drug.

Figure 8 is a schematic representation of the chemical formula of amiloride, HMA and DMA: R=H_2N, amiloride: R=$(CH_3)_2N$, DMA; R .=$(CH_2)_6N$, HMA.

Figure 9A. Channel activity produced by P7 peptide incorporated into artificial lipid bilayers.

Figure 9B. An all points histogram generated from a potential of –80mV in the trans chamber due to channel activity produced by P7 incorporated into artificial lipid bilayers.

Figure 10 is a graphical representation showing current reversal at +50mV for channel activity produced by P7 peptide incorporated into artificial lipid bilayers. Channel activity was measured with 500mV KCl in the cis chamber and 50mM KCl in the trans chamber.

Figure 11. Currents generated over a range of potentials by P7 peptide incorporated into artificial lipid bilayers. Channel activity was measured with 500mM KCl in the cis chamber and 50mM KCl in the trans chamber.

Figure 12. Currents generated over a range of potentials by P7 peptide incorporated into artificial lipid bilayers. Channel activity was measured with 500mM NaCl in the cis chamber and 50mM NaCl in the trans chamber.

Figures 13 and 14. P7 peptide ion channel activity in artificial lipid bilayers is blocked in the presence of hexamethylene amiloride (HMA).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based, in part, on the surprising determination that certain compounds containing a guanidyl moiety are able to inhibit the replication and/or growth of different viruses. Without wishing to be bound by theory or any particular mechanism of action, inhibition of viral replication by the compounds of the present invention may occur via the inhibition of membrane ion channel functioning. Thus, viral infection may cause a host cell to express either an endogenous or a heterologous membrane ion channel, and viral growth/replication may be retarded by inhibiting the functioning of the ion channel with the compounds of the present invention.

Again, without being bound by theory, a number of envelope viruses have been shown to encode small hydrophobic proteins that form ion channels, for example M2 protein of influenza A, Vpu protein of HIV-1 and p7 of hepatitis C virus (HCV). Whereas these ion channels may have important roles in the replicative cycle of viruses, in particular the final stages of virion budding, they have not been utilised for targeting antiviral compounds mostly because of complexity of mechanisms surrounding viral replication and lack of detailed characterisation of their role in this process .

The present observations and findings now permit the use of agents such as, but not limited to, amiloride analogues as anti-viral agents for the therapy and prophylaxis of viral conditions caused by different viruses. The methods and compositions of the present invention may be particularly effective against viruses which rely on ion channel formation for their replication, however it will be understood that this is not the only mechanism relied on by viruses for replication and that the compounds and methods of the present invention are not limited to agents which exert their action by retarding or inhibiting the function of ion channels.

Reference to "membrane ion channel" should be understood as a reference to a structure which transports ions across a membrane. The present invention extends to ion channels which may function by means such as passive, osmotic, active or exchange transport. The ion channel may be formed by intracellular or extracellular means. For example, the ion channel may be an ion channel which is naturally formed by a cell to facilitate its normal functioning. Alternatively, the ion channel may be formed by extracellular means. Extracellular means would include, for example, the formation of ion channels due to introduced chemicals, drugs or other agents such as ionophores or

due to the functional activity of viral proteins encoded by a virus which has entered a cell.

The ion channels which are the subject of certain embodiments of the present invention facilitate the transport of ions across membranes. Said membrane may be any membrane and is not limited to the outer cell wall plasma membrane. Accordingly, "membrane" encompasses the membrane surrounding any cellular organelle, such as the Golgi apparatus and endoplasmic reticulum, the outer cell membrane, the membrane surrounding any foreign antigen which is located within the cell (for example, a viral envelope) or the membrane of a foreign organism which is located extracellularly. The membrane is typically, but not necessarily, composed of a fluid lipid bilayer. The subject ion channel may be of any structure. For example, the Vpu ion channel is formed by Vpu which is an integral membrane protein encoded by HIV-1 which associates with, for example, the Golgi and endoplasmic reticulum membranes of infected cells. Reference hereinafter to "Vpu ion channels" is a reference to all related ion channels for example P7 HCV and M2 of influenza and the like.

Reference to "HIV" or "HCV" should be understood as a reference to any HIV or HCV strain and including homologues and mutants.

Reference to the "functional activity" of an ion channel should be understood as a reference to any one or more of the functions which an ion channel performs or is involved in. For example, the Vpu protein encoded ion channel, in addition to facilitating the transportation of Na^+, K^+, Cl^- and PO_4^{3-}, also plays a role in the degradation of the CD4 molecule in the endoplasmic reticulum. Without wishing to be bound by a particular theory, the Vpu protein encoded ion channel is also thought to play a role in mediating the HIV life cycle. The present invention is not limited to treating HIV infection via the mechanism of inhibiting the HIV life cycle and, in particular, HIV replication. Rather, the present invention should be understood to encompass any mechanism by which the compounds of the present invention exert their anti-viral activity and may include inhibition of HIV viability or functional activity. This also applies to HCV and to other viruses.

Reference to the "functional activity" of a virus should be understood as a reference to any one or more of the functions which a virus performs or is involved in.

Reference to the " viral replication" should be understood to include any one or more stages or aspects of the viral life cycle, such as inhibiting the assembly or release of virions. Ion channel mediation of viral replication may be by direct or indirect means. Said ion channel mediation is by direct means if the ion channel interacts directly with the virion at any one or more of its life cycle stages. Said ion channel mediation is indirect if it interacts with a molecule other than those of the virion, which other molecule either directly or indirectly modulates any one or more aspects or stages of the viral life cycle. Accordingly, the method of the present invention encompasses the mediation of viral replication via the induction of a cascade of steps which lead to the mediation of any one or more aspects or stages of the viral life cycle.

Reference to "down-regulating' ion channel functional activity, should be understood as a reference to the partial or complete inhibition of any one or more aspects of said activity by both direct and indirect mechanisms. For example, a suitable agent may interact directly with an ion channel to prevent replication of a virus or, alternatively, may act indirectly to prevent said replication by, for example, interacting with a molecule other than an ion channel. A further alternative is that said other molecule interacts with and inhibits the activity of the ion channel.

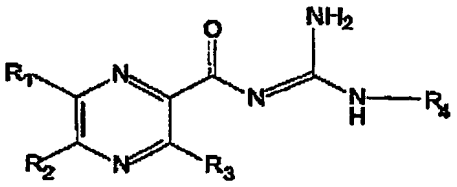
Screening for molecules that have antiviral activity can be achieved by the range of methodologies described herein.

Reference to a "cell" infected with a virus should be understood as a reference to any cell prokaryotic or eukaryotic which has been infected with a virus. This includes, for example, immortal or primary cell lines, bacterial cultures and cells *in situ*. In a suitable screening system for antiviral compounds, the preferred infected cells would be macrophages/monocytes or hepatocytes/lymphoid cells infected with either HIV or HCV respectively.

Without limiting the present invention to any one theory or mode of action, the compounds of the present invention are thought to inhibit viral replication or virion release from cells by causing ion channels, namely VPU of HIV and P7 of HCV to become blocked. This blocking is effected by compounds containing a guanidyl moiety

but not by amiloride itself, despite having the same moiety. The present invention encompasses antiviral compounds comprising a guanidyl moiety and in particular analogues and/or derivatives of amiloride. For example, other isomeric forms of amiloride. Accordingly, reference to "amiloride analogue" should be understood as a reference to any amiloride molecule which has an addition, deletion or substitution, such as an addition, deletion or substitution of an atom or molecule or changing of the charge of an atom or molecule, at any position but more particularly at any one or more of the 6 positions of the pyrazine ring. This definition does not include the compounds 5-(N,N-hexamethylene)amiloride and 5-(N,N-dimethyl)-amiloride so far as they are used in the treatment of HIV infection. Preferably, the amiloride analogues and/or derivatives possesses a substitution of the amino group located at the 5 position and/or the chloride located at the 6-position of the pyrazine, and/or the amino group located at the 3-position of the pyrazine, or one or both of the guanidyl amino groups.

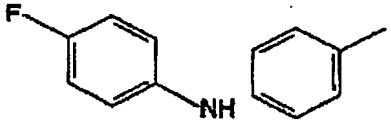
Preferably, the amiloride analogues and/or derivatives of the invention comprise the structure

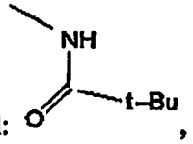


wherein the substituents at the R positions may or maynot be the same, and

R_1 = halo, aryl, substituted aryl, phenyl, or substituted phenyl;

R_2 = amine, aryl, substituted aryl, phenyl, substituted phenyl, hexamethylene, PrS, N-methyl-N-isobutyl, N-ethyl –N-isopropyl, benzyl ; N-methyl-N-guanidinocarbonyl-methyl, N,N-dimethyl, N,N-diethyl, tert-butylamino, halo-aniline, fluoro-aniline



R_3 = hydroxy, alkyloxy, methoxy, N,3-dimethylbutanamyl: 

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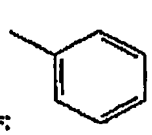
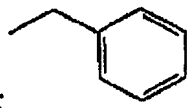
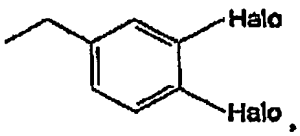
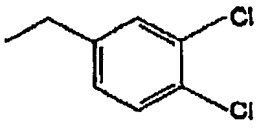
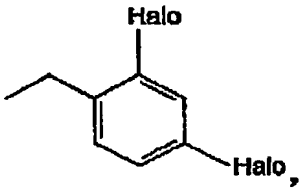
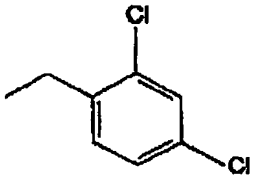
R_4 =:  or  or,  

, — BOD^{IPY_FL};

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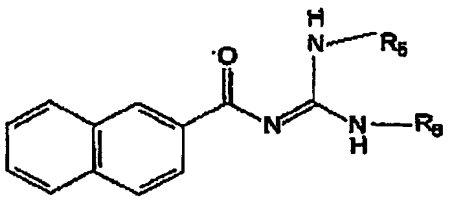
The following antiviral compounds comprising a guanidyl moiety are also encompassed within the scope of the present invention:



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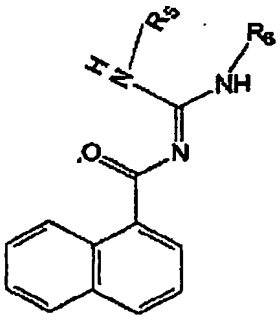

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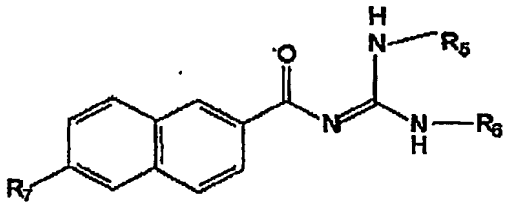
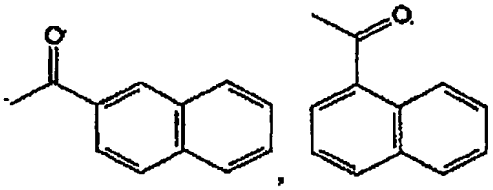


wherein the substitutents at the R positions may or may not be the same, and

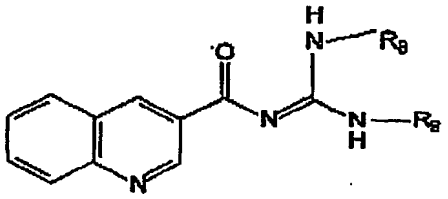
R_5= H, aryl, substituted aryl, phenyl, or substituted phenyl;

R_6 = H, aryl, substituted aryl , phenyl, substituted phenyl, napthoyl,



R_7 = alkyloxy, methoxy;

or, the structure

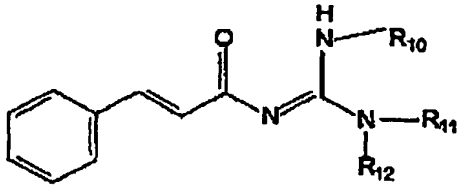


wherein the substitutents at the R positions may or may not be the same, and

R_8 = aliphatic or aromatic substituents;

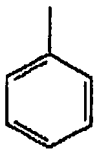
R_9 = aliphatic or aromatic substituents;

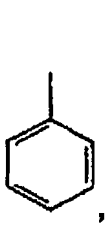
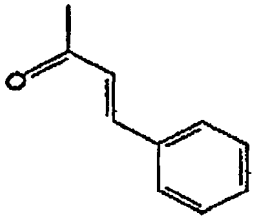
or, the structure



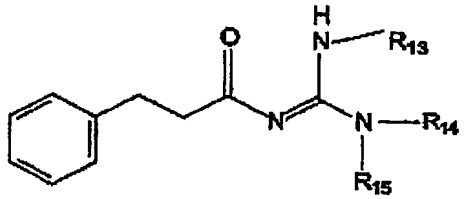
wherein the substitutents at the R positions may or may not be the same, and

R_{10} = H, aryl, phenyl, cinnamoyl;

R_{11} = H, alkyl, aryl, phenyl, cinnamoyl; 

R_{12} = H, alky, aryl, phenyl, cinnamoyl,  , 

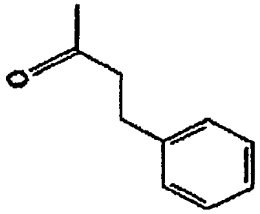
or, the structure



wherein the substitutents at the R positions may or may not be the same, and

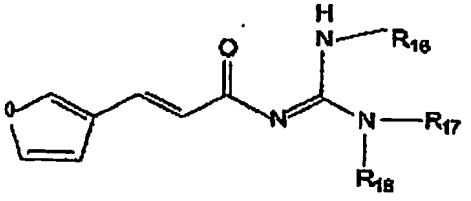
R_{13} = H; alkyl, or phenyl

R_{14} = H, alkyl, phenyl, or substituted phenyl, -



R_{15} = H, or

or, the structure



wherein the substitutents at the R positions may or may not be the same, and

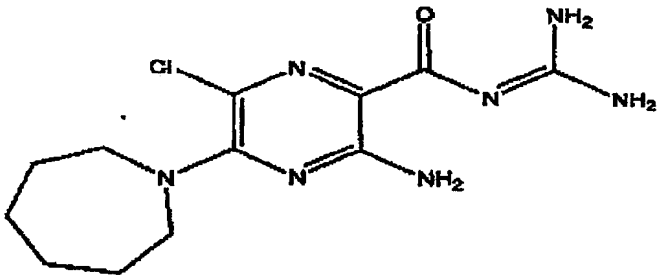
R_{16} = H, alkyl, substituted alkyl, phenyl, or substituted phenyl;

R_{17} = H, alkyl, substituted alkyl, phenyl, or substituted phenyl;

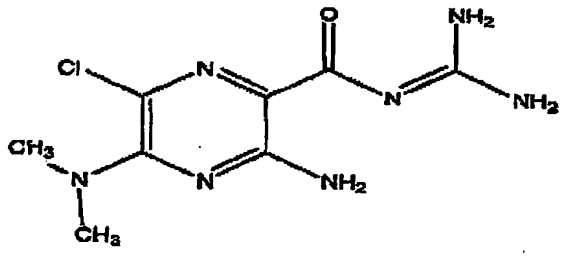
R_{18} = H, alkyl, substituted alkyl, phenyl, or substituted phenyl;

The antiviral compounds of the invention include the following:

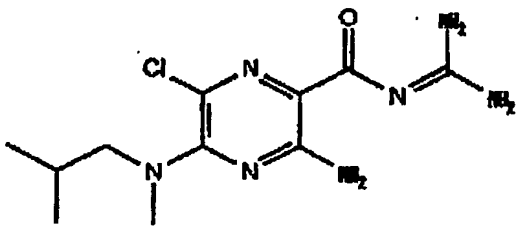
5-(N,N-hexamethylene)amiloride



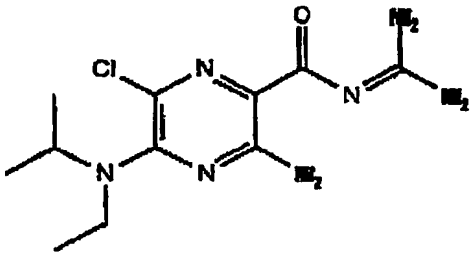
5-(N,N-Dimethyl)amiloride hydrochloride



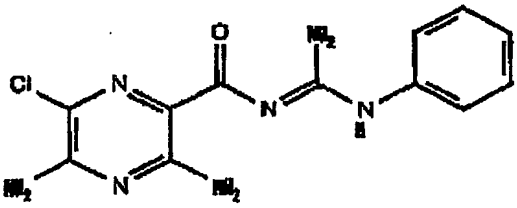
5-(N-methyl-N-isobutyl)amiloride comprising the structure



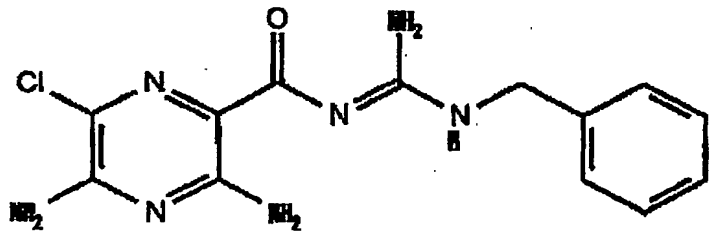
5-(N-ethyl-N-isopropyl)amiloride (herein referred to as EIPA), comprising the structure



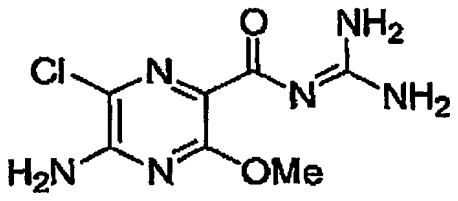
N-(3,5-Diamino-6-chloro-pyrazine-2-carbonyl)-N'-phenyl-guanidine, comprising the structure



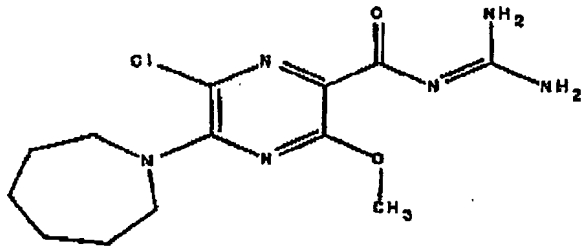
N-Benzyl –N'-(3,5-diamino-6-chloro-pyrzine-2-carbonyl)-guanidine, comprising the structure



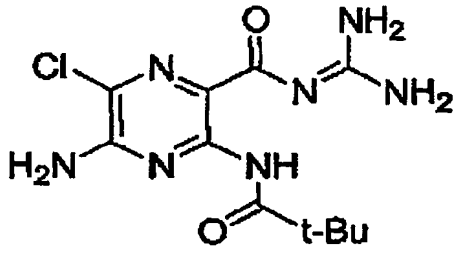
3-methoxy amiloride comprising the structure



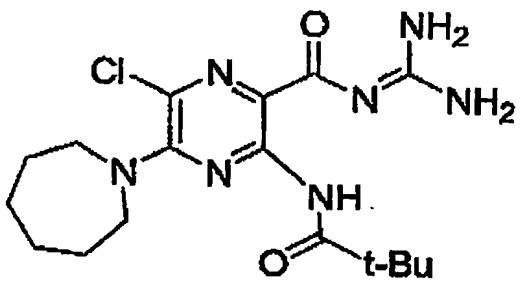
3-methoxy-5-(N,N-Hexamethylene)-amiloride comprising the structure



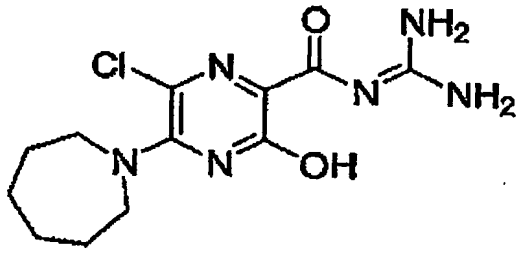
3-(N-2,2 -dimethyl propanal)amiloride comprising the structure



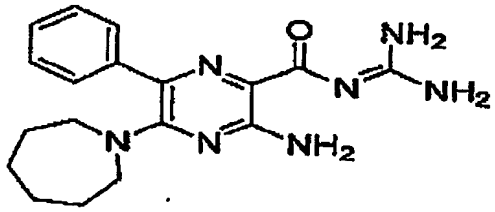
3-(N-2,2 -dimethyl propanal)-5-N-hexamethylene amiloride comprising the structure



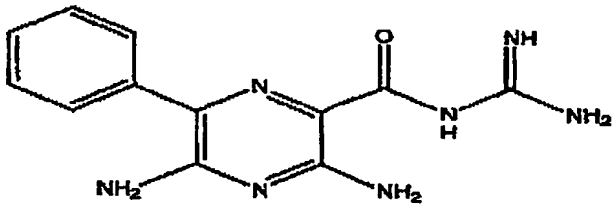
3-hydroxy-5-hexamethyleneimino-amiloride comprising the structure



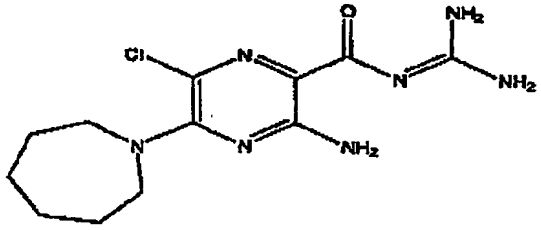
Hexamethyleneimino-6-phenyl-2-pyraxinecarboxamide comprising the structure



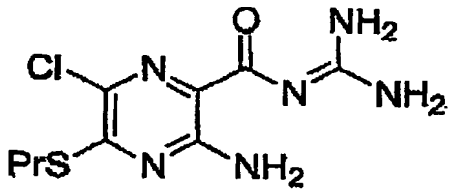
N-amidino-3,5-diamino-6-phenyl-2-pyrazinecarboxamide comprising the structure



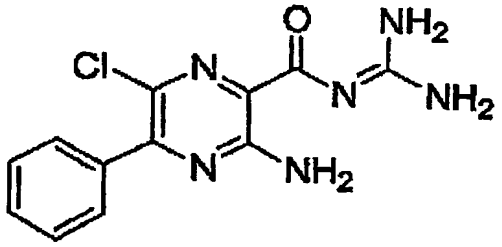
5-(N,N-hexamethylene)amiloride comprising the structure



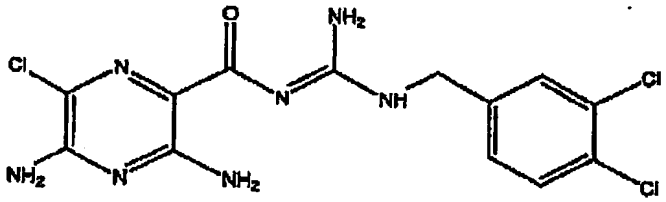
5 5-propyl-sulfide amiloride comprising the structure



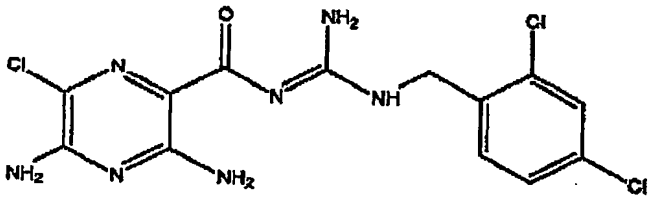
N-amidino-3-amino-5-phenyl-6-chloro-2-pyrazinecarboxamide comprising the structure



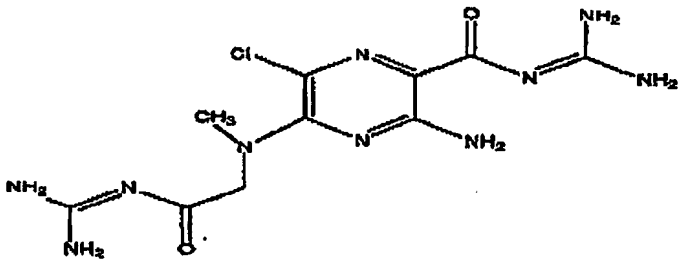
3'4 DichloroBenzamil comprising the structure



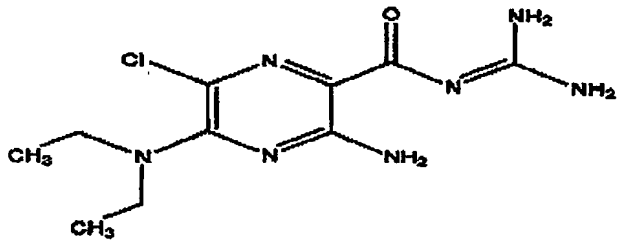
2'4 DichloroBenzamil HCl comprising the structure



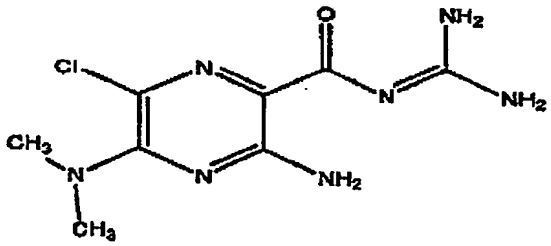
5-(N-methyl-N-guanidinocarbonyl-methyl)amiloride comprising the structure



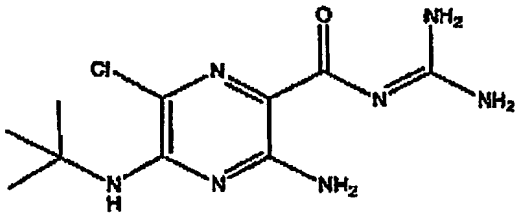
5-(N,N-Diethyl)amiloride hydrochloride comprising the structure



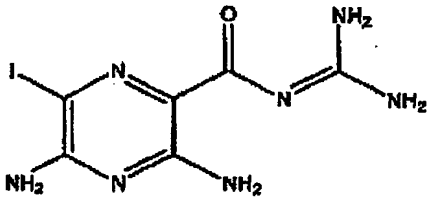
5-(N,N-Dimethyl)amiloride hydrochloride comprising the structure



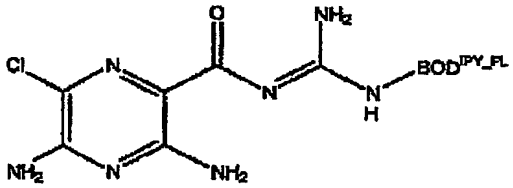
5-tert-butylamino-amiloride comprising the structure



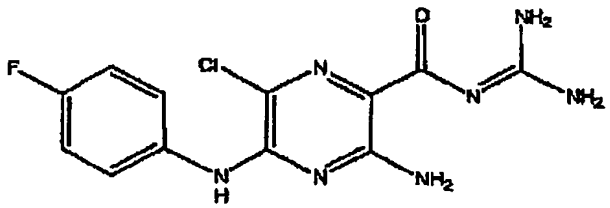
6-Iodoamiloride comprising the structure



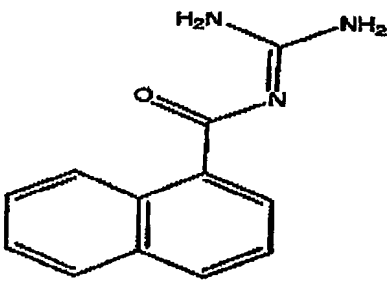
5 Bodipy-FL Amiloride comprising the structure



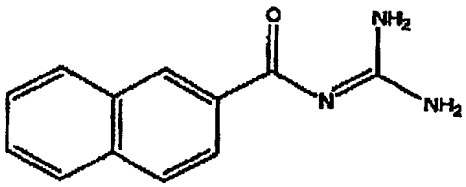
5-(4-fluorophenyl)amiloride comprising the structure



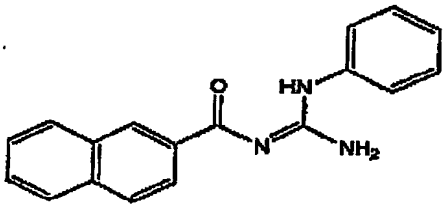
1-napthoylguanidine comprising the structure



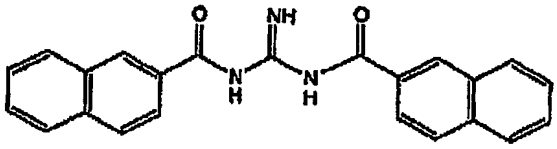
2-napthoylguanidine comprising the structure



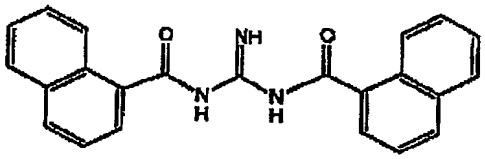
N-(2-napthoyl)-N'-phenylguanidine comprising the structure



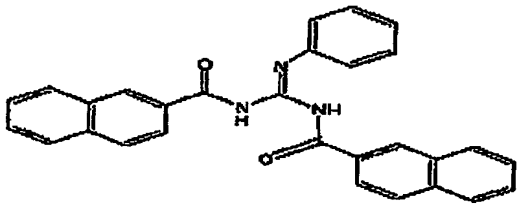
N,N'-bis(2-napthoyl)guanidine comprising the structure



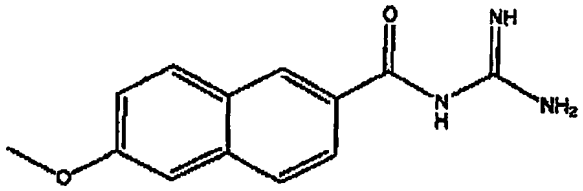
N,N'-bis(1-napthoyl)guanidine comprising the structure



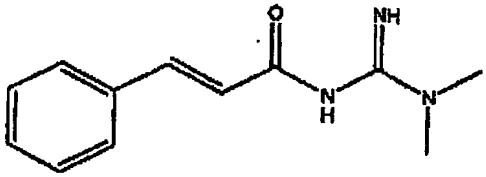
N,N'-bis(2-napthoyl)-N''-phenylguanidine comprising the structure



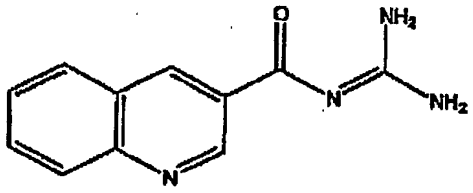
6-methoxy-2-naphthoylguanidine comprising the structure



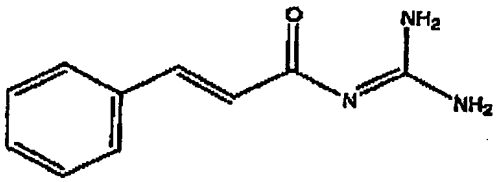
N-Cinnamoyl-N',N'-dimethylguanidine comprising the structure



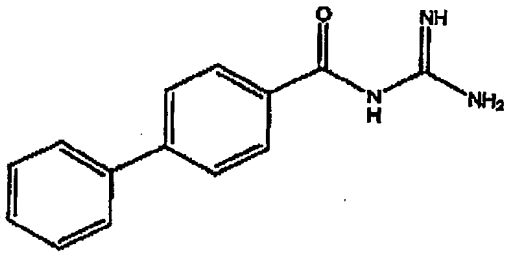
3-quinolinoylguanidine comprising the structure



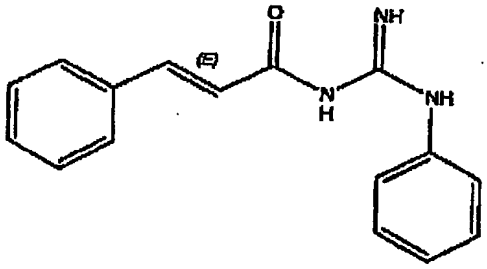
cinnamoylguanidine comprising the structure



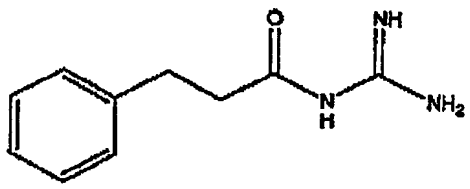
4-phenylbenzoylguanidine comprising the structure



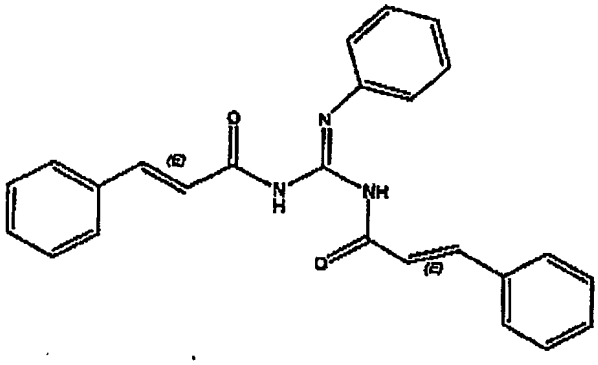
5 N-(cinnamoyl)-N'phenylguanidine comprising the structure



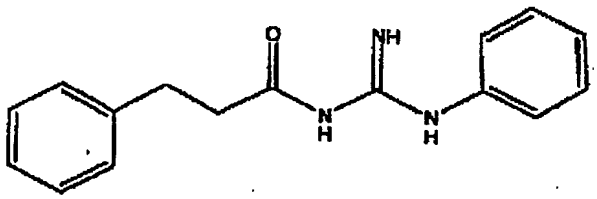
(3-phenylpropanoyl)guanidine comprising the structure



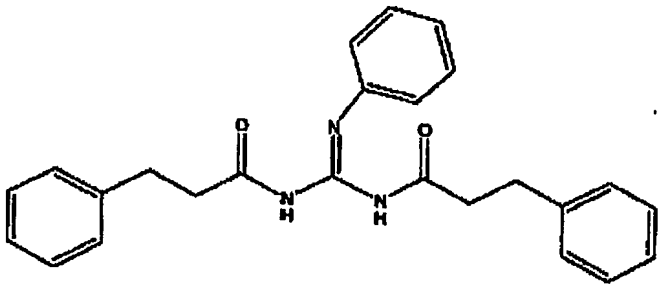
N,N'-bis-(cinnamoyl)-N''-phenylguanidine comprising the structure



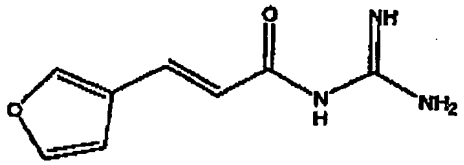
N-(3-phenylpropanoyl)-N'-phenylguanidine comprising the structure



N,N'-bis(3phenylpropanoyl)-N"-phenylguanidine comprising the structure



trans-3-furanacryoylguanidine comprising the structure



or as provided in the following list:

N-(3,5-Diamino-6-chloro-pyrazine-2-carbonyl)-N'-phenyl-guanidine (also referred to as phenamil) (Sigma), N-Benzyl-N'-(3,5-diamino-6-chloro-pyrazine-2-carbonyl)-guanidine (also referred to as Benzamil) (Sigma), 3'4 DichloroBenzamil,2'4 DichloroBenzamil HCl (BioMol CA-204), 5-(N-methyl-N-guanidinocarbonyl-methyl)amiloride, 5-(N-Methyl-N-isobutyl)amiloride (Sigma), 5-(N-Ethyl-N-isopropyl)amiloride (Sigma), 5-(N,N-Dimethyl)amiloride hydrochloride (Sigma), 5-(N,N-hexamethylene)amiloride (Sigma), 5-(N,N-Diethyl)amiloride hydrochloride (Molecular Probes), 6-Iodoamiloride (Molecular Probes), Bodipy-FL Amiloride (Molecular Probes), 3-hydroxy-5-hexamethyleneimino-amiloride, 5-(4-fluorophenyl)amiloride, 5-tert-butylamino-amiloride (Chem. Pharm. Bull., 1997 45, 1282-1286), N-amidino-3-amino-5-phenyl-6-chloro-2-pyrazinecarboxamide, 3-methoxy -HMA, 3-methoxy-amiloride, hexamethyleneimino-6-phenyl-2-pyrazinecarboximide, N-amidino-3-amino-5-hexamethyleneimino-6-phenyl-2-pyrazinecarboxamide, N-amidino-3,5-diamino-6-phynyl-2-pyrazinecarboxamide, 1-napthoylguanidine, 2-napthoylguanidine (Chem.

Pharm. Bull., 1997 45, 1282-1286), N-(2-napthoyl)-N'-phenylguanidine, N,N'-bis(2-napthoyl)guanidine, N,N'-bis(1-napthoyl)guanidine, N,N'-bis(2-napthoyl)-N''-phenylguanidine , 6-methoxy-2-naphthoylguanidine (Chem. Pharm. Bull., 1997 45, 1282-1286), 3-quinolinoylguanidine (Chem. Pharm. Bull., 1997 45, 1282-1286), cinnamoylguanidine (J. Amer. Chem. Soc., 1954, 76, 574-576), 4-phenylbenzoylguanidine, N-(cinnamoyl)-N'phenylguanidine (WO 84/00875), (3-phenylpropanoyl)guanidine (WO 84/00875), N,N'-bis-(cinnamoyl)-N''-phenylguanidine, N-(3-phenylpropanoyl)-N'-phenylguanidine (WO 84/00875), N,N'-bis(3phenylpropanoyl)-N''-phenylguanidine.

Further compounds of the invention include L-cis-diltiazem.HCl (Sigma) and cariporide (Aventis).

The present invention also includes the use of compounds 5-(N,N-hexamethylene)amiloride and 5-(N,N-dimethyl)-amiloride in the control of viral replication and/or growth other than HIV.

The subject of the viral inhibition is generally a mammal such as but not limited to human, primate, livestock animal (e.g. sheep, cow, horse, donkey, pig), companion animal (e.g. dog, cat), laboratory test animal (e.g. mouse, rabbit, rat, guinea pig, hamster), captive wild animal (e.g. fox, deer). Preferably, the subject is a human or primate. Most preferably, the subject is a human.

The method of the present invention is useful in the treatment and prophylaxis of viral infection such as, for example, but not limited to HIV infection, HCV infection and other viral infections. For example, the antiviral activity may be effected in subjects known to be infected with HIV in order to prevent replication of HIV thereby preventing the onset of AIDS. Alternatively, the method of the present invention may be used to reduce serum viral load or to alleviate viral infection symptoms. Similarly, antiviral treatment may be effected in subjects known to be infected with, for example, HCV, in order to prevent replication of HCV, thereby preventing the further hepatocyte involvement and the ultimate degeneration of liver tissue.

The method of the present invention may be particularly useful either in the early stages of viral infection to prevent the establishment of a viral reservoir in affected cells or as a

prophylactic treatment to be applied immediately prior to or for a period after exposure to a possible source of virus.

Reference herein to "therapeutic" and "prophylactic" is to be considered in their broadest contexts. The term "therapeutic" does not necessarily imply that a mammal is treated until total recovery. Similarly, "prophylactic" does not necessarily mean that the subject will not eventually contract a disease condition. Accordingly, therapy and prophylaxis include amelioration of the symptoms of a particular condition or preventing or otherwise reducing the risk of developing a particular condition. The term "prophylaxis" may be considered as reducing the severity of onset of a particular condition. Therapy may also reduce the severity of an existing condition or the frequency of acute attacks.

In accordance with the methods of the present invention, more than one compound or composition may be co-administered with one or more other compounds, such as known anti-viral compounds or molecules. By "co-administered" is meant simultaneous administration in the same formulation or in two different formulations via the same or different routes or sequential administration by the same or different routes. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days between the administration of the two or more separate compounds. The subject antiviral compounds may be administered in any order.

Routes of administration include but are not limited to intravenously, intraperitonealy, subcutaneously, intracranialy, intradermally, intramuscularly, intraocularly, intrathecaly, intracerebrally, intranasally, by infusion, orally, rectally, *via* iv drip, patch and implant. Intravenous routes are particularly preferred.

Compositions suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions. They must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic

acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients, enumerated above, as required, followed by, for example, filter sterilization or sterilization by other appropriate means. Dispersions are also contemplated and these may be prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, a preferred method of preparation includes vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution.

When the active ingredients are suitably protected, they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1 % by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80 % of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 ng and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: A binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint,

oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

The present invention also extends to forms suitable for topical application such as creams, lotions and gels. In such forms, the anti-clotting peptides may need to be modified to permit penetration of the surface barrier. Procedures for the preparation of dosage unit forms and topical preparations are readily available to those skilled in the art from texts such as *Pharmaceutical Handbook. A Martindale Companion Volume Ed. Ainley Wade Nineteenth Edition The Pharmaceutical Press London, CRC Handbook of Chemistry and Physics Ed. Robert C. Weast Ph D. CRC Press Inc.; Goodman and Gilman's; The Pharmacological basis of Therapeutics. Ninth Ed. McGraw Hill; Remington; and The Science and Practice of Pharmacy. Nineteenth Ed. Ed. Alfonso R. Gennaro Mack Publishing Co. Easton Pennsylvania.*

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated

to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved and (b) the limitations inherent in the art of compounding.

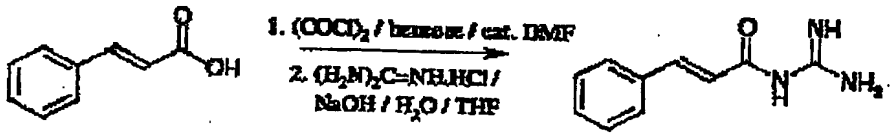
Effective amounts contemplated by the present invention will vary depending on the severity of the pain and the health and age of the recipient. In general terms, effective amounts may vary from 0.01 ng/kg body weight to about 100 mg/kg body weight.

Alternative amounts include for about 0. 1 ng/kg body weight about 100 mg/kg body weight or from 1.0 ng/kg body weight to about 80 mg/kg body weight.

Further features of the present invention are more fully described in the following Examples. It is to be understood, however, that the detailed description is included solely for the purpose of exemplifying the present invention. It should not be understood in any way as a restriction on the broad description of the invention as set out above.

Example 1.

Synthesis of Cinnamoylguanidine from Cinnamic acid Cinnamoyl chloride

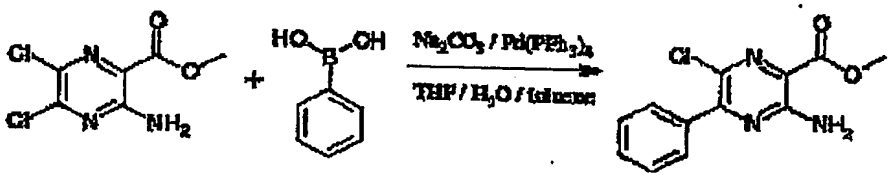


To a solution of *trans*-cinnamic acid (1.50 g, 10.12 mmol) in dry benzene (30mL) containing a drop of *N,N*-dimethylformamide was added oxalyl chloride (5.14 g, 40.5 mmol) causing the solution to effervesce. After refluxing for 2 h, the solution was evaporated to dryness under reduced pressure. The resulting solid was dissolved in dry tetrahydrofuran (20mL) and added slowly to a solution of guanidine hydrochloride in 2M aqueous sodium hydroxide (25mL). The reaction was stirred at room temperature for 1h then extracted with ethyl acetate (3x50mL). The combined extracts were dried over magnesium sulfate and evaporated to give an orange oil. The crude product was purified by column chromatography. Elution with 10% to 20% methanol in dichloromethane gave *Cinnamoylguanidine* as a cream solid (0.829 g, 43%).

Example 2

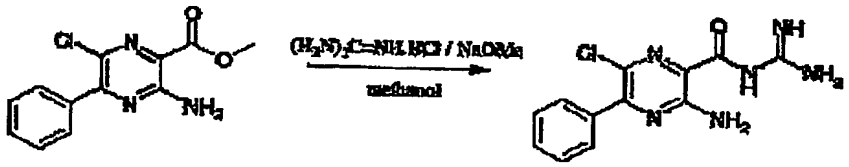
Synthesis of N-amidino-3-amino-5-phenyl-6-chloro-2-pyrazinecarboxamide

Part 1



To a solution of methyl 3-amino-5,6-dichloro-2-pyrazinecarboxylate (0.444 g, 2.0 mmol) in tetrahydrofuran (5 mL) / water (10 mL) / toluene (20 mL) was added phenyl boronic acid (0.536 g, 4.4 mmol), sodium carbonate (0.699 g, 6.6 mmol) and tetrakis(triphenylphosphine)- palladium(0) (0.116 g, 0.10 mmol). The reaction was evacuated and purged with nitrogen several times before being refluxed for 6 h. The organic layer was separated and the aqueous layer extracted with toluene (3 x 20 mL). The combined organic extracts were dried over magnesium sulfate, filtered and evaporated under reduced pressure to give *methyl 3-amino-6-chloro-5-phenyl-2-pyrazinecarboxylate* as a yellow solid (0.43 g, 82%).

Part 2

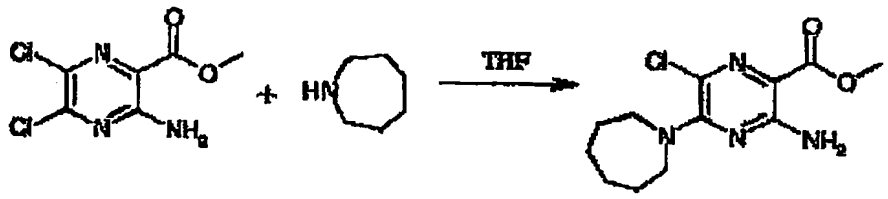


To a solution of sodium (0.040 g, 1.74 mmol) dissolved in methanol (5 mL) was added guanidine hydrochloride (0.258 g, 2.70 mmol) and the mixture refluxed for 30 min after which it was filtered. To the filtrate was added methyl 3-amino-6-chloro-5-phenyl-2-pyrazinecarboxylate (0.264 g, 1.0 mmol) in *N,N*-dimethylformamide (5 mL) and the solution heated at 75°C for 12 h. The solvent was removed under reduced pressure and the residue chromatographed on silica gel eluting with 1% triethylamine / 5% methanol / dichloromethane. The resulting solid was suspended in chloroform, filtered and dried under high vacuum to give *N-Amidino-3-amino-5-phenyl-6-chloro-2-pyrazinecarboxamide* as a yellow solid (0.04 g, 14%).

Example 3.

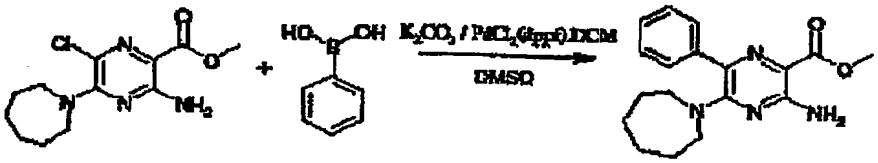
Synthesis of hexamethyleneimino-6-phenyl-2-pyrazinecarboxamide

Part 1



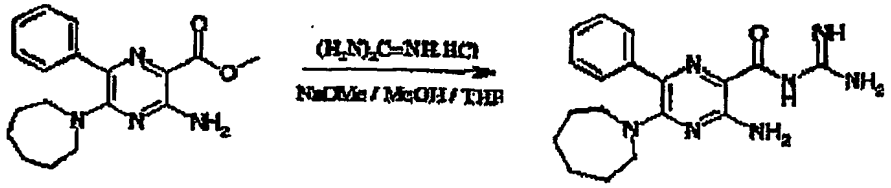
To a solution of methyl 3-amino-5,6-dichloro-2-pyrazinecarboxylate (1.11 g, 5.0 mmol) in tetrahydrofuran (50 mL) was added hexamethyleneimine (1.49 g, 15.0 mmol) and the reaction was refluxed for 1 h. The reaction was allowed to cool and the solid hexamethyleneimine hydrochloride removed by filtration. The filtrate was evaporated and the residue chromatographed over silica gel. Elution with dichloromethane gave *methyl 3-amino-6-chloro-5-hexamethyleneimino-2-pyrazinecarboxylate* as an off-white solid (1.20 g, 85%).

Part 2



To a solution of methyl 3-amino-6-chloro-5-hexamethyleneimino-2-pyrazinecarboxylate (0.350g, 1.23 mmol) in dimethylsulfoxide (5 mL) was added phenyl boronic acid (0.166 g, 1.35 mmol), potassium carbonate (0.511 g, 3.70 mmol) and [1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium(II)-dichloromethane complex (0.041 g, 0.05 mmol). The reaction was heated at 90oC for 16 h before being poured into water (50mL) and extracted with ethyl acetate (3 x 50mL). The combined extracts were dried over magnesium sulfate, filtered and evaporated to give a brown oil which was purified by chromatography on silica gel. Elution with dichloromethane followed by 10% ethyl acetate/dichloromethane gave *methyl 3-amino-5-hexamethyleneimino-6-phenyl-2-pyrazinecarboxylate* as a yellow solid (0.309 g, 77%).

Part 3.



To a solution of sodium (0.090 g, 6.17 mmol) dissolved in methanol (8 mL) was added guanidine hydrochloride (0.598 g, 6.26 mmol) and the mixture was refluxed for 30 min after which it was filtered. To the filtrate was added methyl 3-amino-5-hexamethyleneimino-6-phenyl-2-pyrazinecarboxylate (0.310 g, 0.95 mmol) in tetrahydrofuran (10 mL) and the solution refluxed for 72 h. The solvent was removed under reduced pressure and the residue chromatographed on silica gel. Elution with 5% methanol/dichloromethane gave *N-amidino-3-amino-5-hexamethyleneimino-6-phenyl-2-pyrazinecarboxamide* as a yellow solid (0.116 g, 35%).

Example 4

Construction of recombinant plasmids p2GEXVpu and pPLVpu

The open reading frame encoding Vpu (Fig la) was amplified by PCR from a cDNA 5 clone of an Nde I fragment of the HIV-1 genome (isolate HXB2, McFarlane Burnet Centre, Melbourne, Australia). Native Pfu DNA polyinerase (Stratagene; 0.035 U//l) was chosen to catalyse the PCR reaction to minimise possible PCR introduced errors by virtue of the enzyme's proofreading activity. The 5', sense, primer AGTAGGATCCATGCAACCTATACC (< 400 > 2) introduces a BamH1 site I 0 (underlined) for cloning in-frame with the 3' end of the GST gene in p2GEX (41). This primer also repairs the start codon (bold T replaces a Q of the vpu gene which is a threonine codon in the HXB2 isolate. The 3', antisense, primer TCTGOAATTLTACAGATCAT CAAC (< 400 > 3) introduces an EcoRl site (underlined) to the other end of the PCR product to facilitate cloning. After 30 cycles of 94°C for 45 sec, 55°C for 1 min and 72°C for 1 min in 0.5 ml thin-walled eppendorf tubes in a Perkin-Elmer thermocycler, the 268bp fragment was purified, digested with BamHl and EcoRl and ligated to p2GEX prepared by digestion with the same two enzymes. The resultant recombinant plasmid is illustrated in Fig lb. The entireVpU open reading frame and the BamH1 and EcoRl ligation sites were sequenced by cycle

sequencing, using the Applied Biosystems dye-terminator kit, to confirm the DNA sequence.

To prepare the Vpu open reading frame for insertion into the pPL451 expression plasmid, p2GEXVpu was first digested with BamHI and the 5' base overhang was filled in the Klenow DNA polymerase in the presence of dNTPs. The Vpu-encoding fragment was then liberated by digestion with EcoRI, purified from an agarose gel and ligated into pPL451 which had been digested with HpaI and EcoRI. Western blots subsequently confirmed that the pPLVpu construct (Fig 1c) expressed Vpu after induction of cultures at 42°C to inactivate the cI857 repressor of the PR and PL promoters.

Example 5

Raising polyclonal antibodies for immuno-identification of Vpu

A peptide CALVEMGVEMGHHAPWDVDDL (< 400 > 4) corresponding to the Cterminal 20 amino acid residues of Vpu was synthesised in the Biomolecular Resource Facility (ANU, Australia) using an Applied Biosystems model 477A machine. A multiple antigenic peptide (MAP) was prepared (Lu et al, 1991) by coupling the peptide to a polylysine core via the N-terminal cysteine residue. The MAP was used to immunise rabbits for production of polyclonal antisera recognising the C-terminus of Vpu. For immunisations 1mg of MAP peptide was dissolved in 1.25ml of MTPBS (16mM Na_2HPO_4,4mM $NaHPO^41$ 150mM NaCl pH 7.3) and emulsified with 1.25ml of Freund's complete adjuvant and injected at multiple subcutaneous sites on the rabbit's back. Booster injections used Freund's incomplete adjuvant and were spaced at least 4 weeks apart with serum being sampled 10-14 days after injections.

Example 6

Techniques involving the antibodies

Peptide-specific antibodies were purified from rabbit sera using an Immunopure™ Ag/Ab Immobilisation kit from Pierce. The synthetic peptide was cross-linked via its N-terminal cysteine to the matrix of a 5ml Sulfo Link™ column according to the kit's instructions, 2.5ml of Vpu immunoreactive serum was added to 20ml of Tris buffer (10mM pH 7.4) and passed through the peptide column three times to maximise exposure of the antibodies to the peptide. The column was washed with 20ml of 10mM Tris pH 7.4 followed by 20ml of the same buffer supplemented with 500mM NaCl. The

bound antibodies were eluted in 5ml of 100mM glycine/150mM NaCl, pH 2.5 and eluents were immediately neutralised by addition of 250 µl of 1M Tris pH 9.0 and dialysed overnight against MTPBS.

An anti-Vpu immunoaffinity column was constructed by covalently cross-linking 200 µg of purified antibody to 100µl of protein A agarose beads (Schleicher and Schuell) using the bifunctional cross-linking reagent dimethylpimelimidate as described previously (Harlow and Lane, 1988).

Immunoprecipitation of Vpu was performed by incubation of samples in the presence of approximately 5-fold excess of purified antibody (room temperature for 1 hr) followed by addition of excess protein-A agarose, incubation for 30 min, and centrifugation to pellet the Vpu-antibody complexes. The supernatant, which was subsequently used as a control in the electrophysiological bilayer experiments, was tested by western blotting to confirm that Vpu had been completely removed. Protein samples were electrophoresed on homogeneous 18 % SDS polyacrylamide gels using a minigel apparatus and prepoured gels (Novex). Samples were treated with SDS (3.2 % final) and mercaptoethanol (0. 8 % final) at 60°C for 5 min before loading onto gels. Protein bands were visualised either with Coomassie brilliant blue R250 or by silver staining.

For western blotting, proteins were transferred from acrylamide gels to PVDF membranes using a semi-dry transfer apparatus (Pharmacia LKB). Vpu was detected after consecutive reactions of the blots with polyclonal antiserum or purified antibodies, goat anti-rabbit alkaline phosphatase conjugate and Western Blue™ stabilised substrate (Promega).

Example 7

Purification of Recombinant Vpu from E. Coli.

Cultures of *E. coli* strain XLI-blue cells containing p2GEXVpu were grown at 30°C with vigorous aeration in LB medium supplemented with glucose (6g/L) and ampicillin (50mg/L) to a density of approximately 250 Klett units, at which time IPTG was added to a final concentration of 0.0lmM and growth was continued for a further 4hr. The final culture density was approximately 280 Klett units. Since early experiments revealed that

the majority of expressed GST-Vpu fusion protein was associated with both the cell debris and 30 membrane fractions, the method of Varadhachary and Maloney (Varadhachary and Maloney, 1990) was adopted to isolate osmotically disrupted cell ghosts (combining both cell debris and membrane fractions) for the initial purification steps. Cells were harvested, washed, weighed and resuspended to 10ml/g wet weight in MTPBS containing DTT (ImM) and $MgCl_2$ (10mM). Lysozyme (0.3 mg/ml; chicken egg white; Sigma) was added and incubated on ice for 30 min with gentle agitation followed by 5 min at 37°C. The osmotically sensitised cells were pelleted at 12,000g and resuspended to the original volume in water to burst the cells. The suspension was then made up to lxMTPBS/DTT using a 10x buffer stock and the ghosts were isolated by centrifugation and resuspended in MTPBS/DTT to which was then sequentially added glycerol (to 20 % wt/vol) and CHAPS (to 2 % wt/vol) to give a final volume of one quarter the original volume. This mixture was stirred on ice for 1 hr and then centrifuged at 400,000g for lhr to remove insoluble material. The GST-Vpu fusion protein was purified from the detergent extract by affinity chromatography on a glutathione agarose resin (Sigma). The resin was thoroughly washed in 50mM Tris pH 7.5 containing glycerol (5 %), DTT (lmM), and CHAPS (0.5 %) (Buffer A) and then the Vpu portion of the fusion protein was liberated and eluted from the resin-bound GST by treatment of a 50% (v/v) suspension of the beads with human thrombin (100U/ml; 37°C for lhr). PMSF (0.5mM) was added to the eluant to eliminate any remaining thrombin activity. This Vpu fraction was further purified on a column of MA7Q anion exchange resin attached to a BioRad HPLC and eluted with a linear NaCl gradient (0-2M) in buffer A.

The Vpu was purified to homogeneity - as determined on silver stained gels - on an immunoaffinity column as follows: HPLC fractions containing Vpu were desalted on a NAP 25 column (Pharmacia) into buffer A and then mixed with the antibody-agarose beads for lhr at room temperature. The beads were washed thoroughly and Vpu was eluted by increasing the salt concentration to 2M. Protein was quantitated using the BioRad dye binding assay.

Example 8

Reconstitution of Vpu in Phospholipid Vesicles.

Proteoliposomes containing Vpu were prepared by the detergent dilution method (New, 1990). A mixture of lipids (PE:PC:PS; 5:3:2; lmg total lipid) dissolved in chloroform

was dried under a stream of nitrogen gas and resuspended in 0.1 ml of potassium phosphate buffer (50mM pH 7.4) containing DTT (1mM). A 25µl aliquot containing purified Vpu was added, followed by octylglucoside to a final concentration of 1. 25 % (wt/vol). This mixture was subject to three rounds of freezing in liquid nitrogen, thawing and sonication in a bath type sonicator (20-30 sec) and was then rapidly diluted into 200 volumes of the potassium phosphate buffer. Proteoliposomes were collected by centrifugation at 400,000g for 1hr and resuspended in approximately 150µl of phosphate buffer.

Example 9

Assaying Ion Channel Activity

Purified Vpu was tested for its ability to induce channel activity in planar lipid bilayers using standard techniques as described elsewhere (Miller, 1986; and Piller et al, 1996). The solutions in the CIS and TRANS chambers were separated by a Delrin™ plastic wall containing a small circular hole of approximately 100µm diameter across which a lipid bilayer was painted so as to form a high resistance electrical seal. Bilayers were painted from a mixture (8:2) of palmitoyl-oleoly-phosphatidyl-ethanolamine and palmitoyl-oleolyphosphatidyl-choline (Avanti Polar Lipids, Alabaster, Alabama) in n-decane. The solutions in the two chambers contained MES buffer (10mM, pH 6.0) to which various NaCl or KCl concentrations were added. Currents were recorded with an Axopatch™ 200 amplifier. The electrical potential between the two chambers could be manipulated between +/-200mV (TRANS relative to grounded CIS). Aliquots containing Vpu were added to the CIS chamber either as a detergent solution or after incorporation of the protein into phospholipid vesicles. The chamber was stirred until currents were observed.

Example 10.

Testing the Effect of HMA and DMA on HIV Replication in Human Monocytes and Macrophages.

Human monocytes were isolated from peripheral blood and cultured either for 24hr (one day old monocytes) or for 7 days to allow differentiation into monocyte derived macrophages (MDM). These cells were then exposed to cell-free preparations of HIV isolates and allowed to absorb for 2hr before complete aspiration of the medium, washing once with virus-free medium and resuspension in fresh medium. The cells were

exposed to 50-10μM HMA or DMA either 24 hr prior to infection or after infection. Subsequent HIV replication, at various times after infection, was compared in cells exposed to drugs and in cells not exposed to drugs (controls). The progression and extent of viral replication was assayed using either an HIV DNA PCR method (Fear et al, 1998) or an ELISA method to quantitate p24 in culture supernatants (Kelly et al, 1998).

Example 11

Expression and Purification of Vpu in E.Coli.

The plasmid p2GEXVpu (Fig. 1) was constructed to create an in-frame gene fusion between the GST and Vpu open-reading frames. This system enabled IPTG-inducible expression of the Vpu polypeptide fused to the C-terminus of GST and allowed purification of the fusion protein by affinity chromatography on glutathione agarose.

Optimal levels of GST-Vpu expression were obtained by growing the cultures at 30°C to a cell density of approximately 250-300 Klett units and inducing with low levels of IPTG (0.01mM). To purify the GST-Vpu, a combined cellular fraction containing the cell debris and plasma membrane was prepared by lysozyme treatment of the induced cells followed by a low-speed centrifugation. Approximately 50% of the GST-Vpu protein could be solubilised from this fraction using the zwitterionic detergent CHAPS. Affinity chromatography using glutathione-agarose beads was used to enrich the fusion protein and thrombin was used to cleave the fusion protein at the high affinity thrombin site between the fusion partners, liberating Vpu (Fig. 2A). In fractions eluted from the anion exchange column Vpu was the major protein visible on silver stained gels (Fig. 2B, lane 1). Finally, Vpu was purified to apparent homogeneity on an immunoaffinity column (Fig. 2B, lane 2). The N-terminal amino acid sequence of the protein band (excised from SDS-PAGE gels) corresponding to the immunodetected protein confirmed its identity as Vpu.

Example 12

Vpu Forms Ion Channels in Lipid Bilayers.

To assay for ion-channel formation by Vpu, reconstitution into planar lipid bilayers was performed. When samples (containing between 7 and 70ng of protein) of purified recombinant Vpu were added to the 1ml of buffer in the CIS chamber of the bilayer apparatus, current fluctuations were detected after periods of stirring that varied from 2

to 30 min (Fig. 3). This time taken to observe channel activity approximately correlated with the amount of protein added to the chamber. No channels were detected when control buffer aliquots or control lipid vesicles were added to the CIS chamber. In those control experiments the chambers could be stirred for more than an hour without appearance of channel activity.

Example 13

Properties of The Vpu Channels.

Channel activity was observed in over 40 individual experiments with Vpu samples prepared from five independent purifications. In different experiments, the amplitude of the currents varied over a large range and, again, seemed to approximately correlate with the amount of protein added. The smallest and largest channels measured had conductances of 14 pS and 280 pS, respectively. The channels were consistently smaller when lipid vesicles containing Vpu were prepared and fused to the bilayer rather than when purified protein in detergent solution was added. This may be because the former method included treatment with high concentrations of detergent and a dilution step that may have favoured the breakdown of large aggregates into monomers.

The relationship between current amplitude and voltage was linear and the reversal potential in solutions containing a ten-fold gradient of NaCl (500mM CIS; 50mM TRANS) was +30mV (Fig. 3B). A similar reversal potential was obtained when solutions contained KCl instead of NaCl. In 5 experiments with either NaCl or KCl in the solutions on either side of the membrane, the average reversal potential was 31.0 +/- 1.2mV (+/-SEM). This is more negative than expected for a channel selectively permeable for the cations alone. Using ion activities in the Goldman-Hodgkin-Katz equation gives a P_{Na}/P_{Cl} ratio of about 5.5 indicating that the channels are also permeable to chloride ions. An attempt was made to reduce the anion current by substituting phosphate for chloride ions. When a Na-phosphate gradient (150mM Na^+ & 100mM phosphate CIS; 15mM Na^+ & 10mM phosphate TRANS, pH 6.8) was used instead of the NaCl gradient, the reversal potential was 37.1 +/- 0.2 (+/-SEM, n=2) again indicating a cation/anion permeability ratio of about 5. (For calculations involving the phosphate solutions, the summed activities of the mono and bivalent anions were used and it was assumed that the two species were equally permeable). The current-voltage curve now

exhibited rectification that was not seen in the NaCl solutions. It can be concluded that the channels formed by Vpu are equally permeably to Na^+ and K^+ and are also permeable, though to a lesser extent, to chloride as well as phosphate ions.

Example 14

Bio-Assay for Screening Potential Ion Channel-Blocking Drugs.

As part of a search for drugs that block the Vpu ion channel, a novel bio-assay was developed to facilitate the screening process which would be prohibitively slow if performed in the bilayer assay (Ewart et al, 1996). This bio-assay is based on the observation that expression of Vpu in *E. coli* results in an active Vpu channel located in the plasmalemma that dissipates the transmembrane sodium gradient. As a consequence of this Vpu channel activity, metabolites whose accumulation within the cells is mediated by a sodium dependent co-transporter (for example proline or adenine) leak out of the cell faster than they can be synthesised so that the metabolites' intracellular levels become limiting for growth of the cell. Thereby, an *E. coli* cell expressing Vpu is unable to grow in minimal drop-out media lacking adenine or proline. However, in the presence of a drug that blocks the Vpu channel, the cell is once again able to re-establish its transmembrane sodium gradient - due to the action of other ion pumps in the membrane - and the leakage of metabolites is prevented enabling growth. Experiments to demonstrate that Vpu can form sodium channels in the plasma membrane of *E. coli* were performed as follows.

To express unfused Vpu in E. coli, the vpu open-reading frame was cloned into the plasmid pPL451 (19) to create the recombinant plasmid pPL-Vpu (Fig. 1b). In this vector the strong P_L and P_R lambda promoters are used to drive expression of Vpu under control of the temperature sensitive c1857 represser, such that when grown at 30°C expression is tightly repressed and can be induced by raising the temperature to between 37°C and 42°C. On agar plates, cells containing pPL-Vpu grew when incubated at 30°C and 37°C but not at 42°C, while control strains grew well at 42°C. Liquid cultures of cells containing pPL-Vpu were grown at 30°C to OD_{600}=0.84 then moved to grow at 42°C for two hours (the final cell density was OD_{600}=0.75). The plasma membrane fraction was prepared and western blotting, using an antibody that specifically binds to

the C-terminus of Vpu, detected a single band at approximately 16kDa, indicating that Vpu was expressed and associated with the membranes (Fig. 2A, lane 5).

Example 15

Cross-Feeding Experiments Reveal That Proline Leaks Out of Cells Expressing Vpu.

Uptake of proline by *E. coli* is well characterised and active transport of the amino acid into the cells is known to use the sodium gradient as the energy source (Yamato *et al*, 1994). To detect whether proline leakage occurs, the following cross-feeing assay was used: A lawn of an *E. colt* strain auxotrophic for proline and methionine (Met^- Pro^-), was seeded and poured as a soft agar overlay on minimal drop-out media plates lacking proline but containing methionine. Sterile porous filter discs were inoculated with a Met^+ Pro^+ strain (XL-1 blue) containing either the pPL451 control plasmid or pPL-Vpu and placed onto the soft agar. The plates were then incubated at 37°C or 30°C for two days. After than time a halo growth of the Met^- Pro^- strain was clearly visible surrounding the disc inoculated with the cells containing pPL-Vpu incubated at 37°C (Fig. 4A). This growth can only be due to the leakage of proline from the Vpu-expressing cells on the disc. No such leakage was apparent from the control strain at 37°C nor around either strain on plates grown at 30°C (Fig. 4B).

In contrast to proline transport, the *E. coli* methionine permease is known to belong to the ABC transporter family (Rosen, 1987) and hence be energised by ATP. Identical crossfeeding experiments to those described above were set us except that the Met^- Pro^- strain was spread on minimal drop-out plates lacking methionine but containing proline. No growth of this strain was evident around any of the discs (Fig. 4C), indicating that methionine was not leaking out of the XL-1 blue cells even when Vpu was being expressed.

Example 16

E.Coli Cells Expressing Vpu Require Adenine in the External Medium for Growth.

It was observed that, due to an uncharacterised mutation in the adenine synthesis pathway, growth of *E. coli* cells of the XLl-blue strain expressing Vpu at 37°C was dependant on the presence of adenine in the medium. This allowed the development of an even simpler bioassay for Vpu ion-channel activity than the proline cross-feeding

assay described above: A lawn of XL1-blue cells containing the pPL-Vpu plasmid is seeded onto an agarose plate lacking adenine in the medium, small aliquots of drugs to be tested for inhibition of the Vpu channel are spotted onto the agarose in discrete locations and the plates are incubated at 37°C for a suitable period of time (12-36 hours). Halos of growth around a particular drug application site indicate that the drug has inhibited expression of the Vpu ion channel activity that prevents growth in the absence of the drug.

Example 17

Assay of MIA, EIPA, Phenamil and Benzamil for Vpu Channel Blocking Activity

Amiloride analogues were characterized for their ability to block Vpu ion channel activity reconstituted into planar lipid bilayers. The four compounds tested were, phenamil, 5-(N-methyl-N-isobutyl)amiloride (MIA), benzamil and 5-(N-ethyl-N-isopropyl)amiloride (EIPA). Vpu N-terminal peptide (residues 1-32) dissolved in trifluoroethanol was added to the CIS chamber of the bilayer apparatus and the solutions was stirred until ion currents were observed, indicating incorporation of one or more Vpu ion channels into the bilayer. After recording the channel activity for a few minutes, drugs were added to the solutions in the CIS and TRANS chambers – with stirring - to a final concentration of 100μM. Channel activity was then recorded for at least a further three minutes and the effect of drug addition on ion current was determined by comparing the channel activity before and after drug addition. For each experiment, drug effect was classified into four categories: "Stong block", if current was inhibited approximately 90-100%; "weak block", approx. 50-90% inhibition; partial block, <50%; and "no effect". Experiments were disregarded if currents larger than ±50pA were generated after addition of Vpu N-peptide because in such cases it is possible that non-native peptide aggregates contribute to bilayer breakdown. Such aggregates, by virtue of their disorganized structure may not be specifically blocked by the drugs at the concentrations tested.

Table 3 summarises the results of the bilayer experiments. The chemical structures of the compounds are indicated in Table 3. A novel outcome of these experiments was the strong blocking of Vpu channels observed with Phenamil. Phenamil has a phenyl group derivative at the guanidine group of amiloride. Amiloride itself is not a blocker of Vpu,

whereas addition of the hexamethylene group at the 5- position of the pyrazine ring created a structure (HMA) that blocks the channel at concentrations as low as 25μM. These new results with Phenamil, however, now show that a bulky hydrophobic derivative at the opposite end of the molecule can also turn amiloride into an effective Vpu channel blocker. Interestingly, benzamil, with a very similar structure was much less effective at blocking the Vpu channel. Neither MIA nor EIPA, alternative 5-position derivatives, were found to be as effective as HMA for Vpu inhibition.

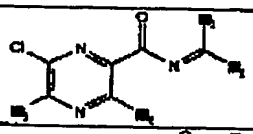
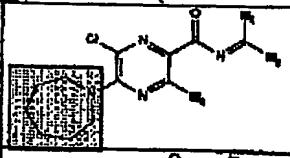
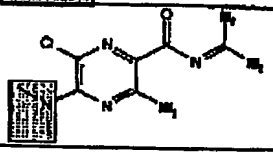
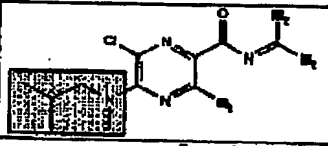
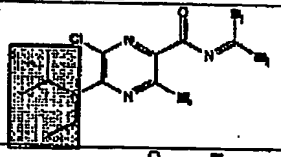
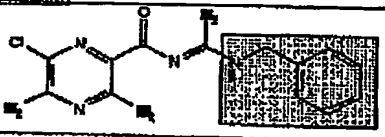
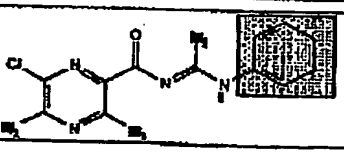
Table 3: Summary of Bilayer Experiments

Compound	No. Expts.	Results
Phenamil	3	3x Strong block
MIA	2	1x Strong block; 1x weak
Benzamil	10	3x partial block; 7x no effect
EIPA	3	3x weak block;
HMA	1	1x Strong block;

Phenamil was subsequently tested in the bacterial growth assay and shown to be an active inhibitor of Vpu function by this method. Table 4 summarises the effectiveness of various amiloride analogues tested in three different assays of Vpu function: the bilayer assay; the bacterial assay; and the anti-HIV assay which assesses the ability of the compounds to inhibit live HIV-1 replication in human blood derived macrophage cells.

Example18:

Table 4: Summary of Effect of Amiloride Analogues in Three Vpu Assays

Compound	Structure	Bilayer Assay	Bacterial Assay	Anti-HIV Assay
Amiloride		Inactive[1]	Inactive[2]	Inactive[4,5]
Hexamethylene Amiloride		Active[1]	Active[3,2]	Active[4,5]
5-(N-N-dimethyl) amiloride		Active[1] (<HMA)	Inactive[3,2]	Active[5]
5-(N-methyl-N-isobutyl)amiloride		Partial activity (at 100μM)	Active[2] (<HMA)	Not Tested
5-(N-ethyl-N-isopropyl)amiloride		Partial activity (at 100μM)	Active[2] (<HMA)	Not Tested
Benzamil		Partial activity (at 100μM – seen in 3/10 experiments)	Not Tested	Not Tested
Phenamil		Strong block (at 100μM seen in 3/3 experiments)	Active (<HMA?)	Not Tested

5

Example 19.

Testing of a Synthetic P7 Peptide for channel activity in artificial lipid bilayers

A peptide mimicking the protein P7 encoded by the hepatitis C virus (HCV) was synthesised having the following amino acid sequence:

ALENLVILNAASLAGTHGLVSFLVFFCFAWYLKGRWVPGAVYAFYGMWPLL LLLLALPQRAYA

Lipid bilayer studies were performed as described elsewhere (Miller, 1986). A lipid mixture of palmitoyl-oleoyl-phosphatidylethanolamine, palmitoyl-oleoyl-phosphatidylserine and palmitoyl-oleoyl-phosphatidylcholine (5:3:2) (Avanti Polar Lipids, Alabaster, Alabama) was used. The lipid mixture was painted onto an aperture of 150-200 um in the wall of a 1 ml delrin cup. The aperture separates two chambers, cis and trans, both containing salt solutions at different concentrations. The cis chamber was connected to ground and the trans chamber to the input of an Axopatch 200 amplifier. Normally the cis chamber contained 500 mM KCl and the trans 50 mM KCl. The bilayer formation was monitored electrically by the amplitude of the current pulse generated by a current ramp. The potentials were measured in the trans chamber with respect to the cis. The protein was added to the cis chamber and stirred until channel activity was seen. The currents were filtered at 1000 Hz, digitized at 2000 Hz and stored on magnetic disk. The P7 peptide was dissolved in 2,2,2-trifluorethanol (TFE) at 10mg/ml. 10 ul of this was added to the cis chamber of the bilayer which was stirred. Channel activity was seen within 15-20 min.

When the P7 peptide was added to the cis chamber and stirred, channel activity was recorded. An example of this channel activity is shown in Fig 9A. The potential in the trans chamber was -80 mV and the currents are downwards. The currents reversed at +50 mV (Fig 10), close to the potassium equilibrium potential in these solutions indicating that the channels were cation-selective. An all-points histogram of a longer record (14.2 seconds) at this potential in the same experiment is shown in Fig 9B. The amplitude of the open-channel peak is 1.7 pA corresponding to a channel conductance of about 14 pS. In most experiments, "single channels" had a much larger size, presumably because of aggregation of the P7 peptide. An example

of these currents over a range of potentials is shown in Fig. 11. The currents reversed at about +40 mV in this experiment. In some experiments the solution in the cis chamber was 150 mM KCl and 15 mM KCl in the trans chamber. The P7 peptide again produced currents that reversed.

Similar results were obtained when both chambers contained NaCl. Currents recorded in an experiment when the cis chamber contained 500 mM NaCl and the trans chamber 50 mM NaCl are shown in Fig 12. Again the currents reversed between +40 and +60 mV, close to the Na^+ equilibrium potential indicating that channels were much more permeable to Na^+ than to K^+.

The channels formed by the P7 peptide were blocked by HMA, as illustrated in Fig 13. The traces in Fig 13A were obtained at 0 mV: the cis chamber contained 500 mM KCl and the trans 50 mM KCl.

Addition of the P7 peptide produced the channel activity shown in Fig 13A. Addition of 2 µl of 50 mM HMA to the cis chamber followed by stirring resulted in disappearance of the channel activity as illustrated in the traces in Fig 13B. Block of channel activity produced by the P7 peptide with 100 µM HMA was recorded in 4 experiments. In 2 experiments, sodium channels (500/50) were blocked by 500 µM HMA

When 10 mM $CaCl_2$ was added to the cis chamber (K solutions) the reversal potential of the currents produced by P7 peptide shifted to more negative potentials indicating a decrease in the P_K/P_{Cl} ratio.

When the cis chamber contained 500 mM $CaCl_2$ and the trans chamber 50 mM $CaCl_2$, both positive and negative currents were seen at potentials around +20 mV and it was not possible to determine a reversal potential.

Example 20.

Recombinant Expression of HCV p7 protein.

Two cDNA fragments, each encoding the same polypeptide corresponding to the amino acid sequence of the HCV-1a p7 protein, were synthesised commercially by GeneScript. The two cDNAs differed in nucleotide sequence such that in one cDNA ("cDp7.coli") the codons were optimised for expression of the p7 protein in E.coli while in the other cDNA ("cDp7.mam)" codons were biased for expression in mammalian cell lines. cDp7.coli was cloned into the plasmid pPL451 as a BamHI/EcoRI fragment for expression in E.coli (see Example 21). cDp7.mam was cloned into vectors (for example, pcDNA3.1 vaccinia virus, pfastBac-1) for expression of p7 in mammalian cell lines (see Example 22).

Example 21

Role of p7 in enhancement of Gag VLP Budding.

The budding of virus-like particles (VLP) from cultured HeLa cells results from the expression of retroviral Gag proteins in the cells and co-expression of small viral ion channels, such as M2, Vpu and 6K, with the Gag protein enhances budding. Interestingly, the viral ion channels can enhance budding of heterologous virus particles. Therefore, to assess budding enhancement by p7it was co-expressed with the HIV-1 Gag protein in HeLa cells, and VLP release into the culture medium was measured by Gag ELISA. To achieve this, the plasmids pcDNAp7 (pc DNA3.1 = pcDp7.mam as described in Example 20, p7 expressed under control of the T7 promoter) and pcDNAGag (HIV-1 Gag protein expressed under control of the T7 promoter) were cotransfected into HeLa cells infected with the vaccinia virus strain vTF7.3 (expresses T7 RNA polymerase) and culture supernatants were collected for ELISA assay after 16 hours incubation.

Example 22

HCV p7 Ion Channel inhibits Bacterial Cell growth.

Analogously to the bio-assay of Vpu ion channel activity described in Examples 14-16, a bio-assay of p7 function in bacterial cells was also developed. The p7-encoding synthetic cDNA fragment cDp7.coli was cloned into the expression plasmid pPL451, creating the vector pPLp7, in which p7 expression is temperature inducible, as

described in Example 4. Inhibition of the growth of E.coli cells expressing p7 at 37°C was observed as an indicator of p7 ion channel function dissipating the normal Na+ gradient maintained by the bacterial cells.

Example 23

Assay of the ability of amiloride analogues to inhibit p7 ion channel functional activity.

The three methods of detecting p7 ion channel functional activity, described in Examples 21-23, were employed to assay the ability of compounds to inhibit the p7 channel. In the case of Example 21, compounds were tested for their ability to inhibit p7 channel activity in planar lipid bilayers. In the case of Example 22 compounds were tested for their ability to reduce the number of VLPs released from cells expressing both p7 and HIV-1 Gag. In the case of Example 23, compounds were tested for their ability to restore growth on minimal nutrient media of E.coli cells expressing p7.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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DATED this 26th Day of June 2003
BALDWIN SHELSTON WATERS
Attorneys for:BIOTRON LIMITED.

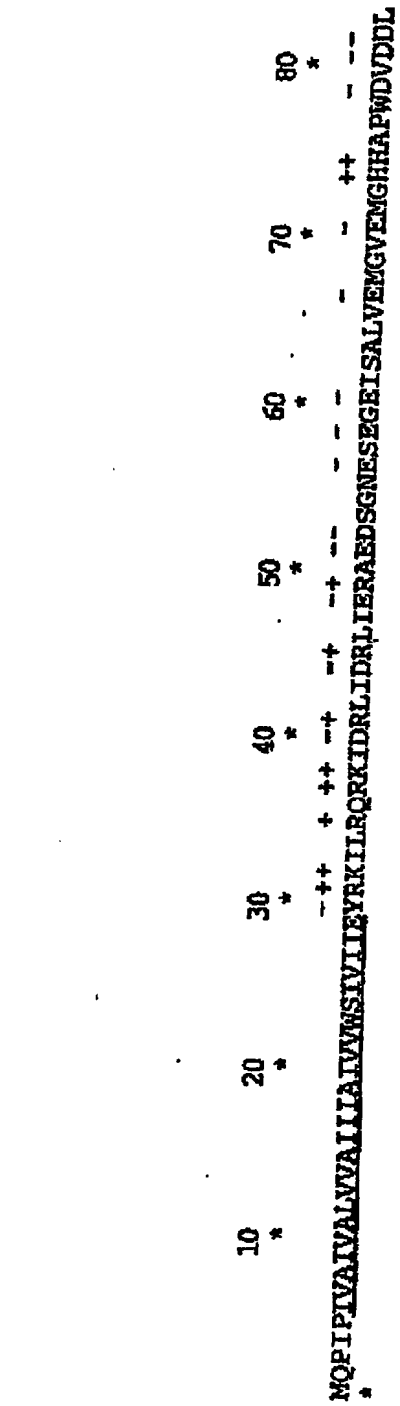
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Figure 1a

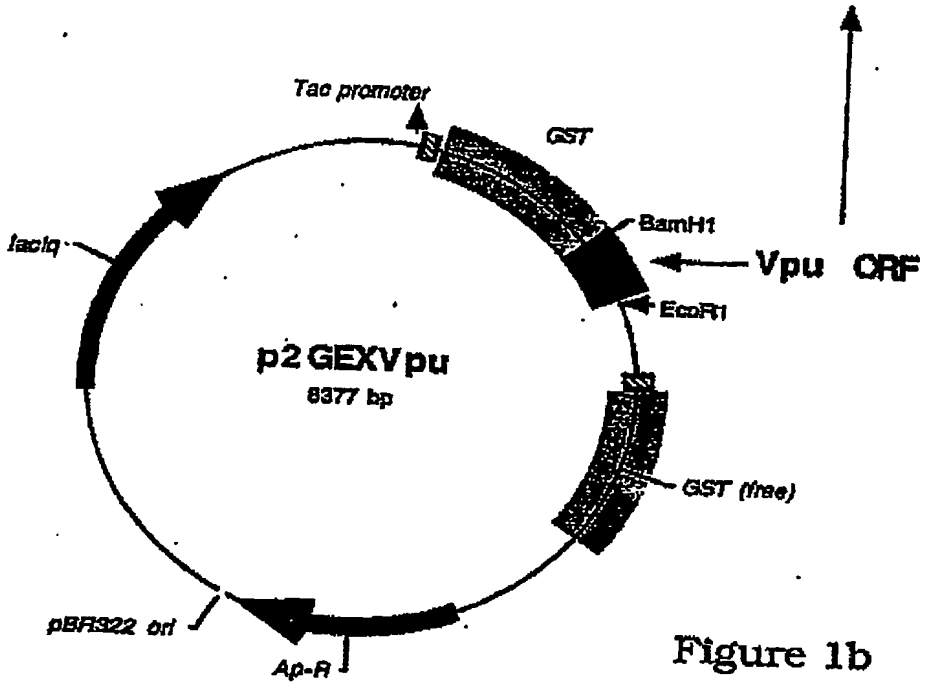


Figure 1b

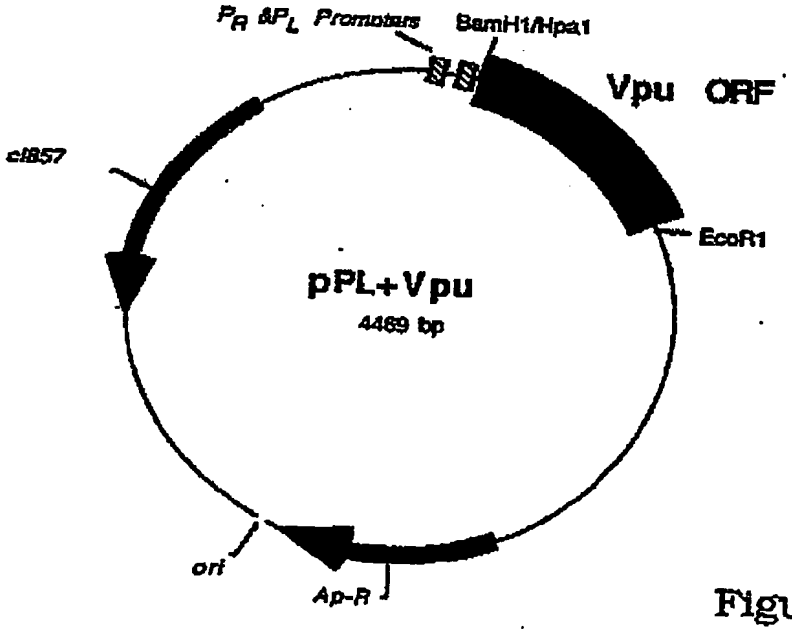


Figure 1c

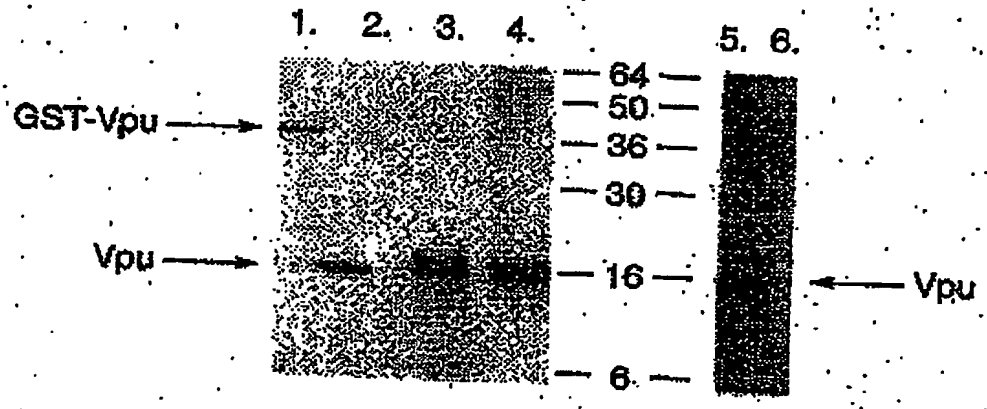


Figure 2a

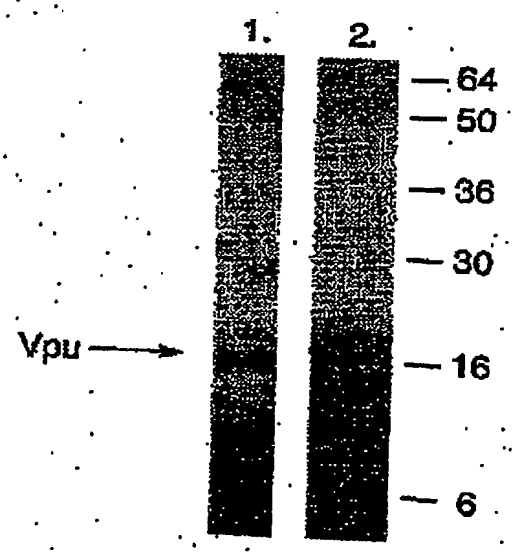


Figure 2b

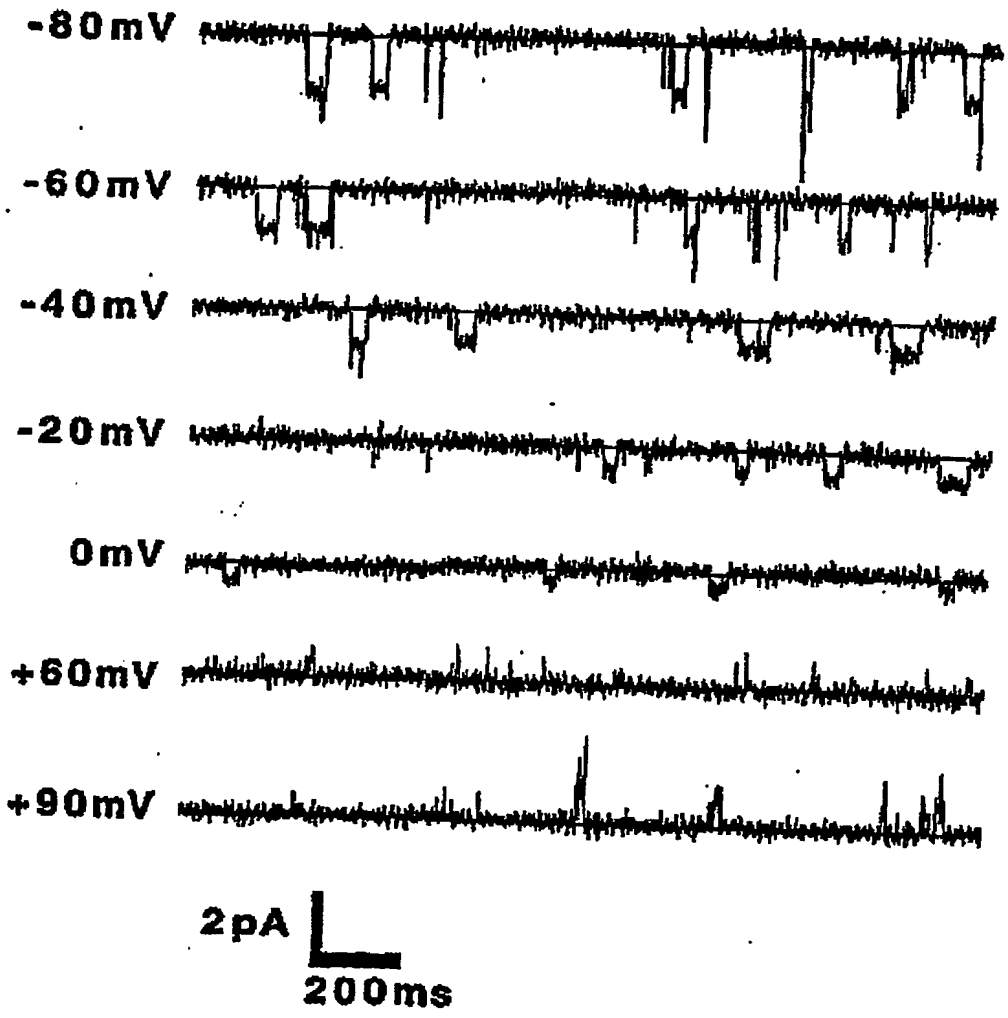


Figure 3a

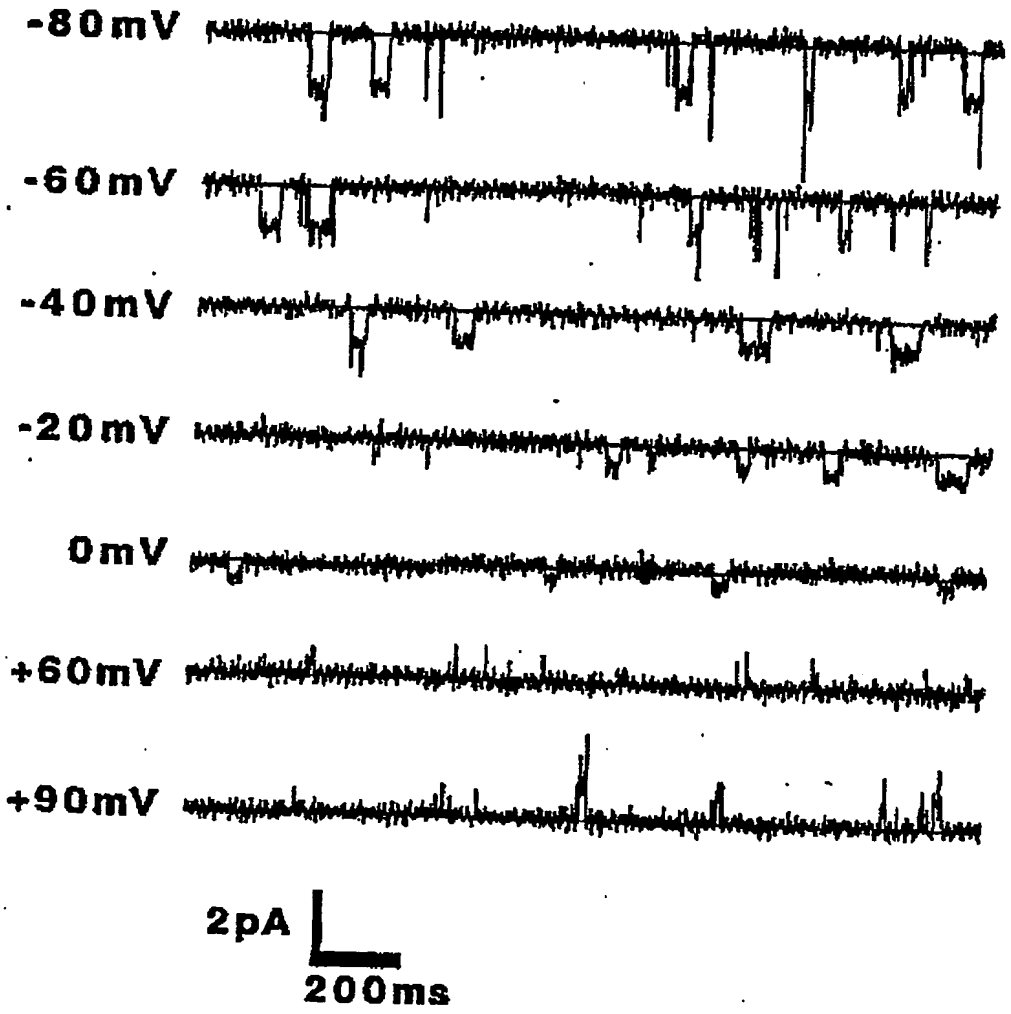


Figure 3a

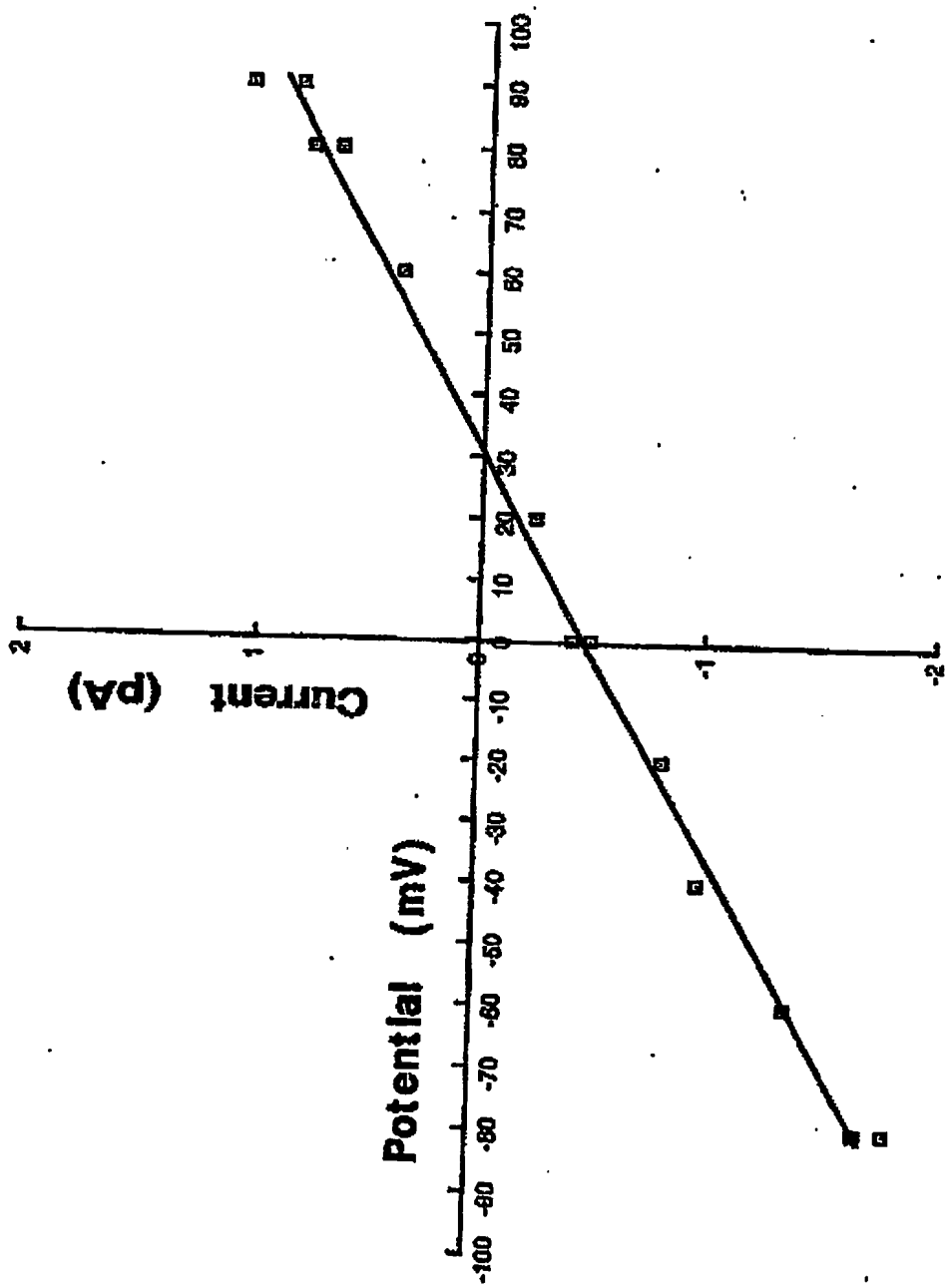
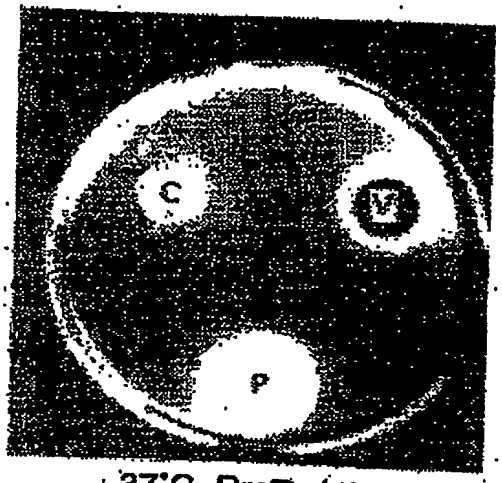

Current (pA)
Potential (mV)
2
1
0
-1
-2
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100

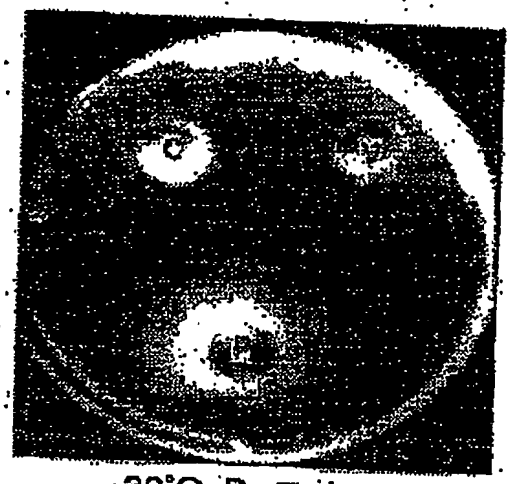
Figure 3b

Figure 4a



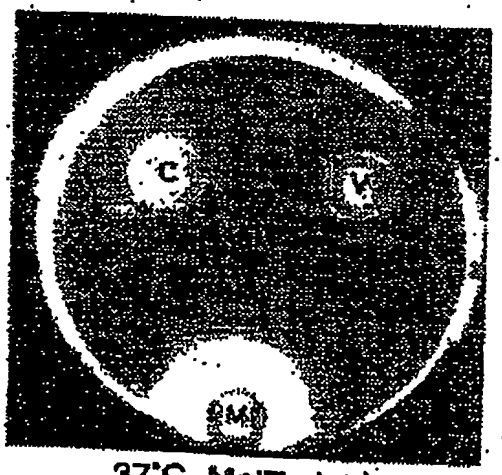
37°C, Pro⁻ plate

Figure 4b



30°C, Pro⁻ plate

Figure 4c



37°C, Met⁻ plate

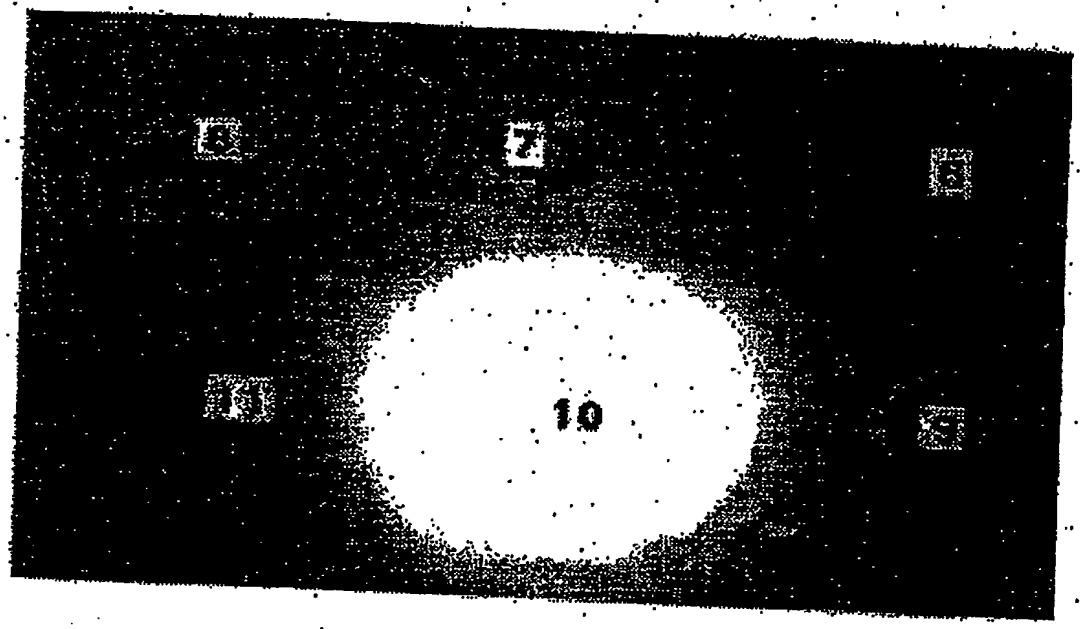
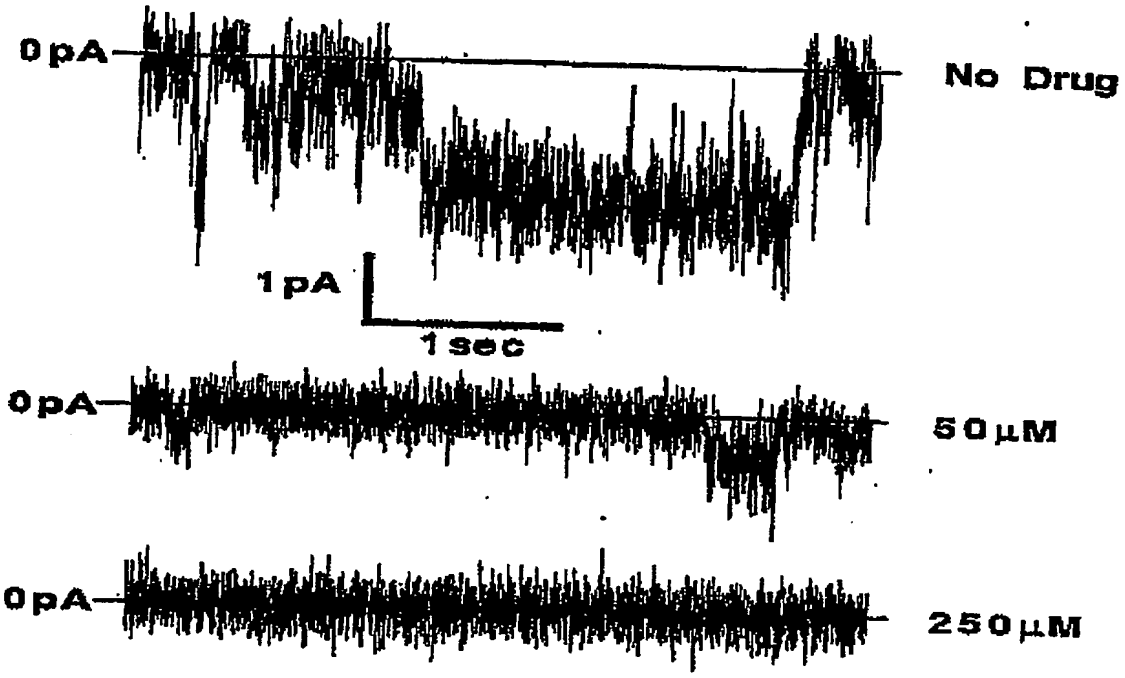


Figure 5



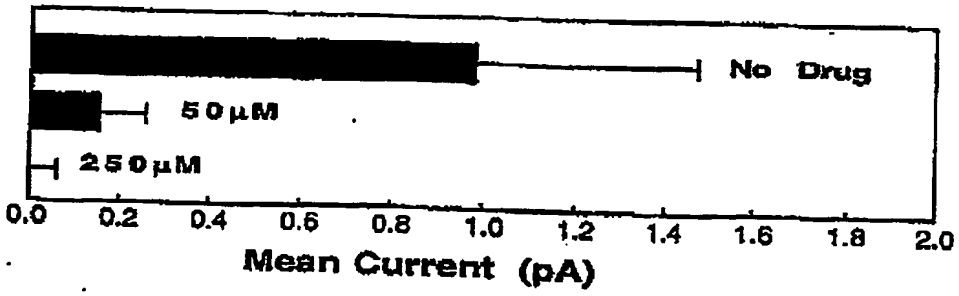
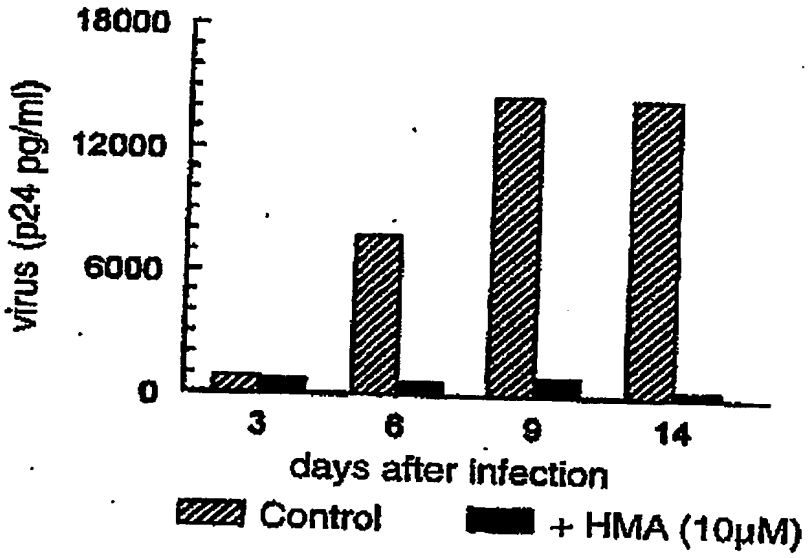


Figure 6

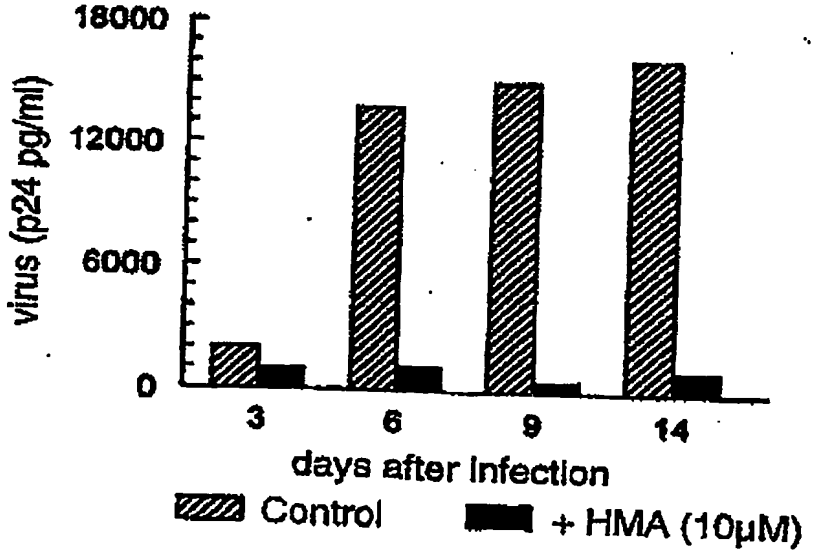
Monocytes

Figure 7a



Macrophages

Figure 7b



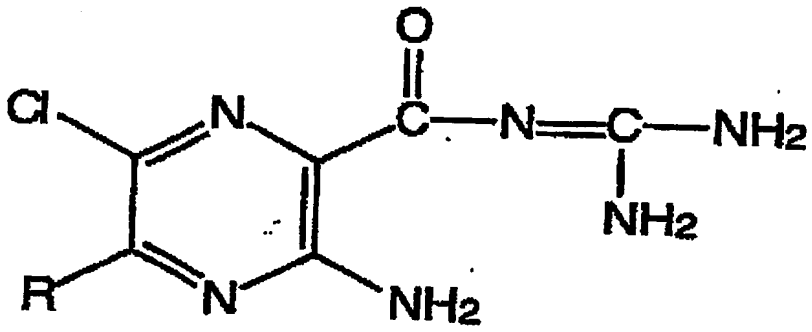
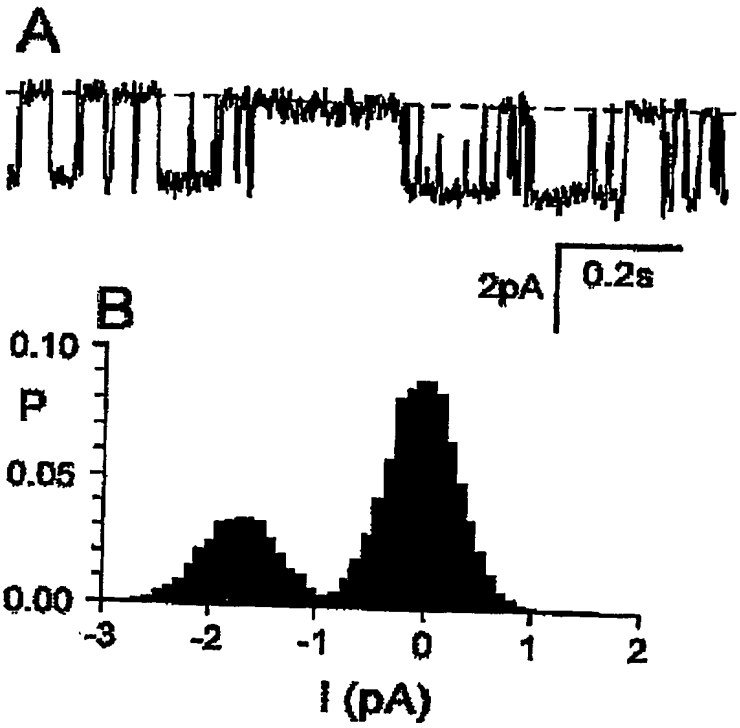


Figure 8



ap0011 -80mV HCV P7

Figure 9

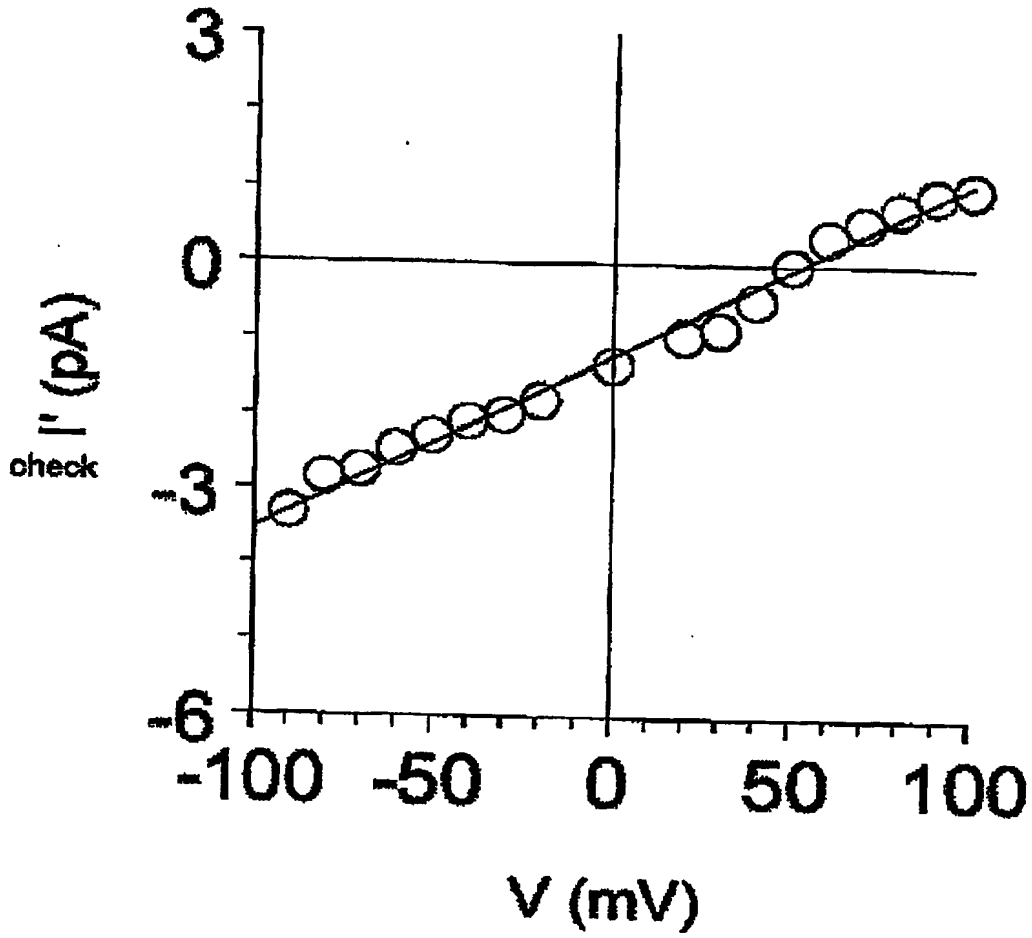


Figure 10

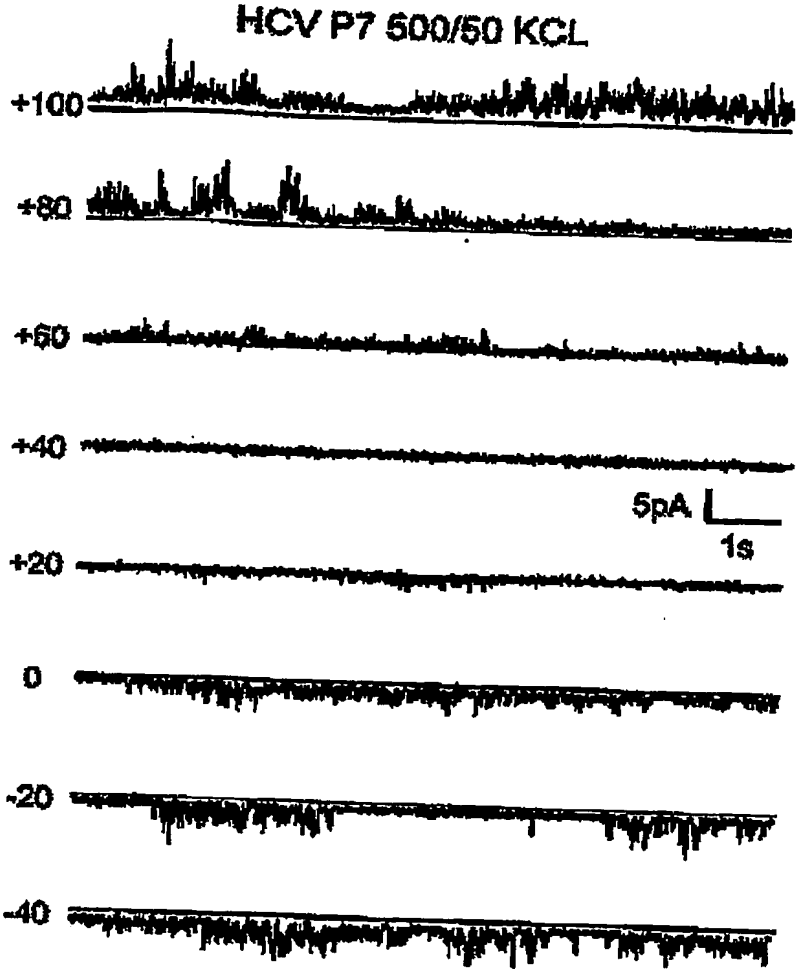


Figure 11

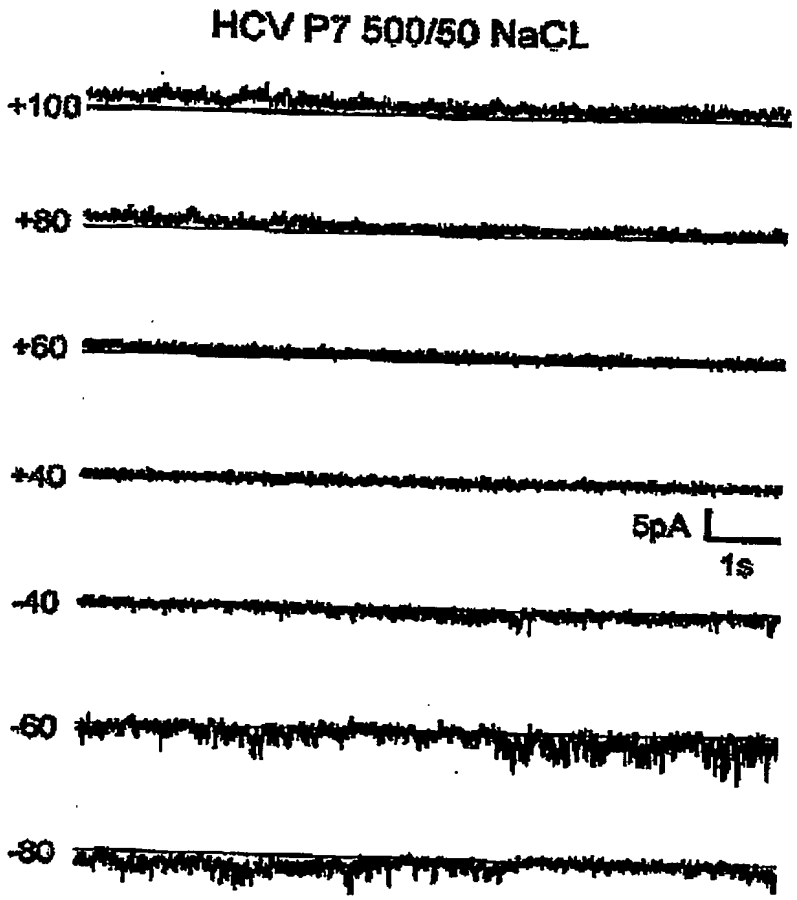


Figure 12

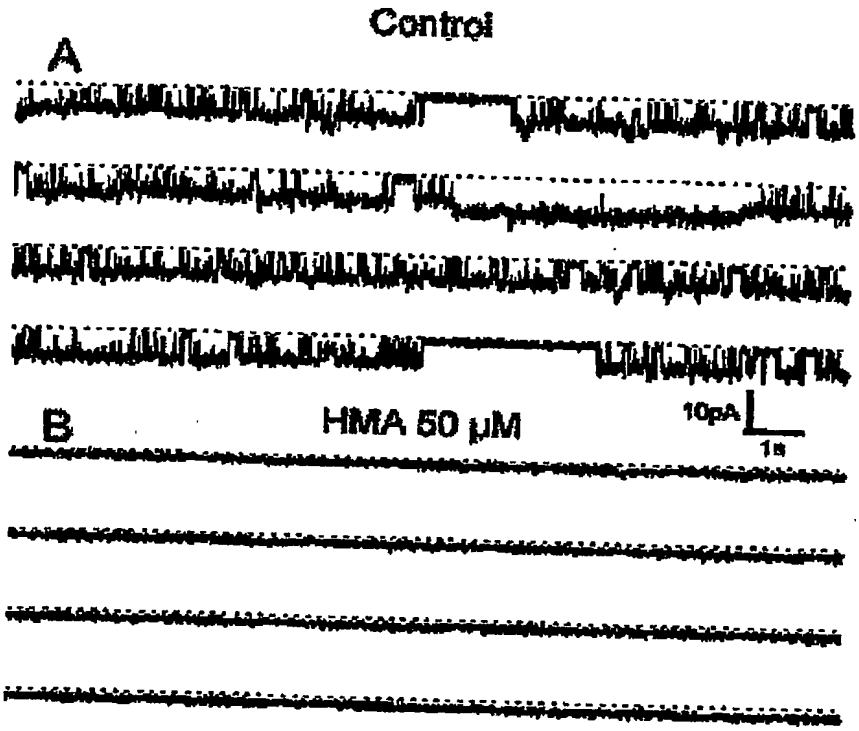


Figure 13

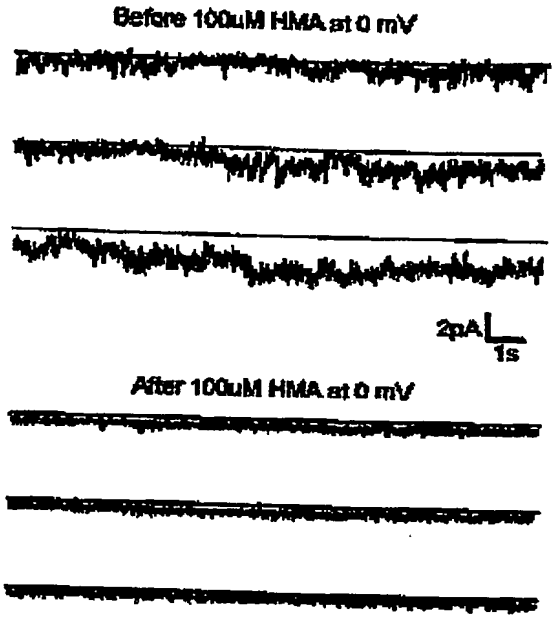


Figure 14